



STATE OF ISRAEL

REC'D 25 JUN 1999

WIPO PCT

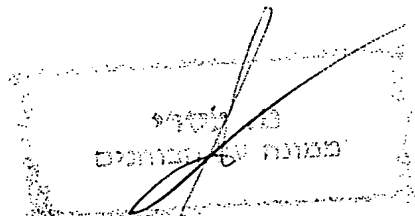
This is to certify that annexed hereto is a true copy of the documents as originally deposited with the patent application particulars of which are specified on the first page of the annex.

זאת לתעודה כי  
רצופים בזה העתקים  
נכונים של המסמכים  
שהופקדו לכתחילה  
עם הבקשה לפטנט  
לפי הפרטים הרשומים  
בעמוד הראשון של  
הנספח.

## PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

This 18-05-1999 היום



רשם הפטנטים  
Registrar of Patents

ג'שה ב'וכ'מ'ת לאבחון נט'ה לאפ'לפס'ה ונ'טור ט'פול נוגד אפ'לפס'ה

**BIOCHEMICAL APPROACH TO DIAGNOSIS OF PREDISPOSITION TO  
EPILEPSY AND MONITORING OF ANTIEPILEPTIC TREATMENT**

מספר: 124444	תאריך: 12-05-1998
Ante/Post-dated	

# ב ק ש ה ל פ ט נ ט Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)  
I (Name and address of applicant, and in case of body corporate - place of incorporation)

1. Svetlana Dolina  
14/12 Aharoni, Rehovot 76282
2. Aaron Rabinkov  
19/8 Bnei Moshe, Rehovot 76485
3. Eugene Pressman  
21/22 Hadadi Zion Shaul, Bat Yam 59514

1. סבטלנה דולינה  
אהרונים 14/12, רחובות 76282
2. אהרון רבינקוב  
בני משה, רחובות 76485
3. יוג'ין פרסמן  
הדדי ציון שאול 21/22, בת ים 59514

בעל אמצאה מכח היותי הממציא ..... Being the Inventor  
of an invention the title of which is ..... Owner, by virtue of

גישה ביוכימית לאבחון נטיה לאפילפסיה וניטור טיפול נוגד אפילפסיה (בעברית)  
(Hebrew)

BIOCHEMICAL APPROACH TO DIAGNOSIS OF PREDISPOSITION TO (באנגלית)  
EPILEPSY AND MONITORING OF ANTIEPILEPTIC TREATMENT (English)

hereby apply for a patent to be granted to me in respect thereof

מבקש בזאת כי ינתן לי עליה פטנט

• בקשת חלוקה - Application of Division		• בקשת פטנט מוסף - Application for Patent Addition		• דרישת דין קדימה Priority Claim	
מבקשת פטנט from Application	לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האיגוד Convention Country	
No. .... dated .....	No. .... dated .....				
P.O.A.: general/individual-attached/to be filed later- הוגש בענין..... filed in case .....					
המען למסירת מכתבים בישראל Address for service in Israel DR. MARK FRIEDMAN LTD. BEIT SAMUELOFF 7 HAOMANIM STREET 67897 TEL AVIV					
חתימת המבקש Signature of Applicant		היום 12 ..... בחודש May ..... שנת 1998 of the year of This			
		לשימוש הלשכה For Office Use			

Ref.: 1067/1

טופס זה כשהוא מוטבע בחותם לשכת הפטנטים ומושלם במספר ובתאריך ההגשה, הנו אישור להגשת הבקשה שפרטיה רשומים לעיל.  
This form, impressed with the seal of the Patent Office and indicating the number and date of filing, certifies the filing of the application the particulars of which are set out above.

Delete whatever is inapplicable מחק את המיותר

# BIOCHEMICAL APPROACH TO DIAGNOSIS OF PREDISPOSITION TO EPILEPSY AND MONITORING OF ANTIEPILEPTIC TREATMENT

## FIELD AND BACKGROUND

5           The present invention is of methods and diagnostic assays for a biochemical approach to the detection of a predisposition to epilepsy, to the clarification of the diagnosis of epilepsy, for improved monitoring of the treatment regimen for antiepileptic drugs, and for distinguishing between pyridoxine dependency and pyridoxine deficiency.

10           Epilepsy is one of the most common chronic neurological disorders. The disease is characterized by recurrent seizures, which originate from abnormal and excessive activity of cerebral neurons and result in a paroxysmal disorganisation of brain function.

          Types of epilepsy include partial (symptomatic) and generalized  
15    idiopathic seizures. Partial epilepsy is "localization related" and originates in a limited area of the brain. The generalized form of epilepsy is not caused by a specific brain lesion or disease, other than a possible genetic propensity to generate seizures. Generalized, or *grand mal*, seizures include tonic-clonic seizures, in which the entire body undergoes convulsions. Left untreated,  
20    epilepsy can degenerate into status epilepticus, a potentially fatal neurological emergency [*Antiepileptic Drugs*; eds. R.H. Levy, R.H. Mattson and B.S. Meldrum; 4th Edition, Raven Press, NY, NY; Y.Aicardi, *Epilepsy in children*, 2d Edition, Press 1994].

Idiopathic epilepsy appears to be a heritable disorder though little is known about the precise genetic or biochemical defects involved (Andermann, 1982; Delgado - Escueta et al, 1986, Anderson, 1982; Anderson et al., 1986; see attached Appendix for complete references). Recent research has indicated the possibility of genetic predisposition to the development of localization-related epilepsy, in particular post-traumatic epilepsy. In this type of epilepsy, a head injury is the resolving exogenous factor inducing the disease with a low penetration of the pathological hereditary factor.

Over 53 million people world-wide suffer from epilepsy, with 2.5 million who have had, or who will have seizures at some point in the US alone. Epilepsy primarily affects children and young adults. Almost 50% of new epilepsy cases occur prior to age 25. A large number of children and adults have undetected or untreated epilepsy.

Early diagnosis of epilepsy is crucial, since repeated seizures may cause severe brain damage and intellectual deterioration. Learning difficulties, behaviour disturbances and poor social adjustment are associated with epileptic syndromes.

Unfortunately, no predictive *in vitro* diagnostic test is currently available. At present, diagnosis of epilepsy is based primarily on clinical history, EEG paroxysmal activity and findings on neuroimaging when available. A sensitive, reliable, and non-invasive method for pre-symptomatic diagnosis of epilepsy would undoubtedly benefit individuals in certain groups at high risk of developing epilepsy. Such groups include first and second degree relatives of

epileptic patients, babies born prematurely or with a traumatic delivery, febrile convulsive children and children with attention deficit disorder. About 80% of children in the last group have underdiagnosed epilepsy according to computer analysis of EEG records. Others at risk include those patients who have endured  
5 severe cerebral injury or intracranial infections, pregnant women, soldiers or pilots who will be subjected to stressful situations, among others. If available, *in vitro* pre-symptomatic diagnosis would provide an opportunity in the future for prophylactic treatment for members of these groups at high risk of developing epilepsy, preferably based upon the suggested pathogenetic  
10 mechanism.

Antiepileptic drugs (AED) are used to reduce the number and the severity of seizures in patients with epilepsy. Serious side effects are associated with the treatment of epilepsy by currently used AED, with adverse reactions occurring in up to 50% of epileptic patients. Of those patients who are free of  
15 seizures because of drug therapy, 31% have complained of memory loss, difficulty in thinking clearly, drowsiness, behavioural changes, clumsiness, lethargy and weight gain. Many patients suffer from epilepsy which is refractory to antiepileptic drugs. Refractory epilepsy accounts for about 30% of the non-compliance cases, of which 15% cannot achieve full seizure control and 15% do  
20 not receive any benefit at all from AED treatment.

Currently available methods for monitoring of AED treatment are not sufficiently sensitive or accurate, as they only rely upon the measurement of the

concentrations of AED in the blood. Instead, a diagnostic approach is required for the detection of biochemical markers indicating seizure susceptibility.

One known mechanism resulting in seizures involves problems with the concentration and metabolism of vitamin B6. Vitamin B6 plays a crucial role in the metabolism of amino acids, proteins, carbohydrates, lipids, hormones and neuromediators. The catalytically active form, pyridoxal-5'-phosphate (PLP; a total list of abbreviations may be found within the "Summary of the Invention" below), is the coenzyme of a large number of enzymes in mammalian tissues, including transaminases, decarboxylases and lyases, etc. Neurotransmitters (dopamine, norepinephrine, serotonin, tyramine, tryptamine, taurine, GABA ( $\gamma$ -aminobutyric acid), and indirectly acetylcholine) are also synthesized and/or metabolized by PLP-dependant enzymatic reactions (Metzler, 1977; Leklem & Reynolds, 1988 for reviews ).

For several decades, primary dietary vitamin B6 (pyridoxine) deficiency has been known to induce convulsions. The erroneous use of a vitamin B6-deficient commercial infant formula induced large number of convulsions in infants in the USA in the early 1950's (May, 1954). The first attempts for defining vitamin B6 requirements in infants were made after this epidemic of vitamin B6 deficient convulsions.

In addition to pyridoxine deficiency, another form of vitamin B6 disorder, pyridoxine dependency, have been described. Pyridoxine dependency is an autosomal recessive genetic disorder, which causes severe convulsions with subsequent mental retardation in neonates and infants (Scriver et al., 1989).

A reduction in the synthesis of GABA, catalyzed by PLP-dependent glutamic acid decarboxylase, is considered to be the main cause for pyridoxine dependent convulsions (Scriver et al., 1989).

Our previous experiments carried out in our proprietary animal model of genetic epilepsy, inbred EP (epilepsy prone) and ER (epilepsy resistant) sublines of the BALB/c strain of mice, have shown that EP animals differ from ER by

- substantially increased susceptibility to seizures;
- lower sensitivity to thiosemicarbazide, the direct pyridoxine antagonist;
- 10       - increase in the pyridoxine-modifiable ratio of glutamate to GABA in all of the brain regions which were examined;
- intensified turnover of phosphatidylinositol (PI) in the brain plasma membranes; and
- cortical serotonin deficiency .

15       Experiments with persistent high dose pyridoxine treatment (75-100 mg/L in drinking water) started in EP animals at the embryonic stage have shown that pyridoxine-treated EP animals differ from untreated EP animals by the following characteristics:

- high tolerance to pentamethylentetrazol (PTZ)-induced and sound-  
20   induced convulsions;
- abolished imbalances in the glutamate/GABA ratio present in specific regions of the brain;
- normalized PI turnover in the plasma membranes of brain tissue; and



- restored serotonin cortical deficiency.

Taken together, these data suggest that an inborn error in vitamin B6 metabolism may trigger a neurotransmitter imbalance and membrane instability, thus causing enhanced susceptibility to convulsions which is characteristic of EP animals (Dolina, Kozak; 1987, Dolina et al., 1993). The multiplicity of biochemical systems affected in EP animals which are corrected by constant high-dose pyridoxine treatment also suggests that a general impairment of vitamin B6 metabolism (rather than a defect in any single PLP-dependent enzyme) induces this wide range of disorders shown in EP animals.

The kynurenine pathway of tryptophan metabolism, as shown in Figure 1, is known to be pyridoxal 5'-phosphate (PLP)-dependent. Kynureninase (EC 3.7.1.3), the enzyme which catalyzes the synthesis of both AA (anthranilic acid) from L-KYN (kynurenine) and 3HOAA (3-hydroxyanthranilic acid) from 3HOKYN (3-hydroxykynurenine), is especially sensitive to the supply of PLP (Bender, 1989). A reduction in kynureninase activity with increased formation of xanthurenic acid as a response to tryptophan loading has been used as a test for deficient vitamin B6 nutritional status. Another PLP-dependent enzyme of the kynurenine pathway (with much higher  $K_m$ ) is KAT (kynurenine aminotransferase (EC 2.6.1.7)), which transforms L-KYN to kynurenic acid (KA), and 3-hydroxykynurenine (3HOKYN) to xanthurenic acid as well. Thus, there are at least two points along the kynurenine pathway of tryptophan degradation which are sensitive to the disorders in the vitamin B6 metabolism.

Disturbed tryptophan metabolism was first reported in children with epilepsy more than thirty years ago (French et al., 1965). For example, the urinary excretion of xanthurenic acid after tryptophan loading (100 mg/kg orally) was found to be abnormally high in two-thirds of the children with cryptogenic epilepsy. Abnormally high levels of xanthurenic acid and 3HOAA have also been obtained in the urine of children with infantile spasm, the most severe form of epilepsy.

Recently, neuroactive properties of the metabolites of the kynurenine pathway – L-KYN, KA, AA, 3HOKYN, 3HOAA, QUIN - have attracted considerable attention as endogenous neuroprotective and neurotoxic agents in the number of neurological diseases and in epilepsy as well.

Kynurenic acid (KA) is an excitatory amino acid receptor antagonist with antineurotoxic and anticonvulsant activity. KA effectively blocks the action of NMDA, quinolinic acid, quisqualate and kainic acid in the rat neocortex, hippocampus, n. caudate and spinal cord (Perkins and Stone, 1982; Stone, 1993). The concentration of KA in the blood and extracellular compartment is regulated by the availability of L-KYN. L-KYN effectively crosses the blood-brain barrier (Fukui et al., 1991), and produces a linear dose-dependent increase in KA concentration (Stone 1993). A relatively low concentration of L-KYN and KA in the CSF (cerebro-spinal fluid) was found both in patients with infantile spasm (Yamamoto, 1991; Yamamoto et al., 1995) and intractable complex partial seizures (Young et al., 1983; Heyes et al. 1994).

By contrast to KA, quinolinic acid (QUIN), an excitotoxic agonist of NMDA (N-methyl-D-aspartate) receptor, is the most potent endogenous convulsant discovered so far (Stone, 1993). QUIN is synthesized by the enzyme 3HAO (3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6)) from the precursor 3HOAA, and is catabolized by quinolinic acid phosphoribosyltransferase (QPRT; EC 3.2.2.5) within the brain. The activity of these enzymes controls the level of extracellular QUIN. QUIN is thought to be unable to penetrate the blood-brain barrier. Once extracellular levels exceed a critical limit, QUIN may enter brain cells via passive diffusion (Schwarcz et al., 1991). High levels of both 3HOKYN and QUIN were found in the CFS of patients with infantile spasm.

3-hydroxyanthranilic acid (3HOAA), which is formed by kynureninase from 3-hydroxykynurenine, is an immediate and efficient precursor of QUIN which mimics the neurotoxic effect of QUIN. 3HOAA also does not cross the blood-brain-barrier. 3HOAA may produce neurotoxicity not only through a direct conversion to QUIN, but also by autooxidation to several products (superoxide, hydrogen peroxide, hyperoxyl and anthranyl radicals) with a potential for toxicity.

According to our assumption, an inborn error in vitamin B6 metabolism may form a genetic basis of an enhanced convulsibility. Therefore, the altered concentrations of the metabolites of PLP-dependent kynurenine pathway can be used as the biochemical markers of the genetic predisposition to epilepsy. Being changed under AED treatment, these markers in combination with the

measurement of the concentration of AED in the blood can be used for the effective monitoring of antiepileptic therapy. However, such a use for the detection of the altered concentrations of metabolites has never been taught or suggested by the prior art.

5

#### SUMMARY OF THE INVENTION

It is one object of the present invention to provide a biochemical approach for the detection of a genetic predisposition to epilepsy and for the clarification of the diagnosis of clinical epilepsy itself, for example to confirm  
10 the diagnosis of clinical epilepsy already made by another approach. In particular, the present invention features the detection of the altered biochemical profile of tryptophan metabolites of the kynurenine pathway, which is associated both with clinical epilepsy and with the genetic predisposition to epilepsy.

15 It is another object of the present invention to provide improved monitoring of epilepsy treatment by AED (antiepileptic drugs).

It is still another object of the present invention to provide a diagnostic system implementing a biochemical approach to pre-symptomatic detection of a genetic predisposition to epilepsy, to the clarification of the diagnosis of clinical  
20 epilepsy and to monitoring of epilepsy treatment by AED.

These and other objects of the present invention will be further detailed in the following description, Figures and claims.

Disturbances in the metabolism of tryptophan by the kynurenine pathway are found in individuals with clinical epilepsy and with a genetic predisposition to epilepsy. These metabolic disturbances result in alterations to the concentrations of individual kynurenine metabolites, as well as to the ratio of certain metabolites. These alterations can be used as biochemical markers for the detection of a predisposition to epilepsy, the clarification of the diagnosis of clinical epilepsy, monitoring of treatment with anti-epileptic drugs, and distinguishing between pyridoxine dependent and pyridoxine deficient conditions.

According to the present invention, there is provided a method for diagnosing a predisposition to epilepsy, or epilepsy itself, in a subject, the method comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least one kynurenine metabolite in the sample; and (c) comparing the concentration of the at least one kynurenine metabolite in the sample to a range of values of the concentration of the at least one kynurenine metabolite for normal individuals, such that if the concentration of the at least one kynurenine metabolite in the sample lies outside of the range of values for normal individuals, the presence of epilepsy in the subject is diagnosed.

Preferably, the sample is a blood sample. Alternatively and preferably, the sample is a urine sample, or any liquid or tissue sample. More preferably, the at least one metabolite is selected from the group consisting of KYN (kynurenine), 3HOKYN (3-hydroxykynurenine), AA (anthranilic acid), 3HOAA

(3-hydroxyanthranilic acid), KA (kynurenic acid) and QUIN (quinolinic acid).

Most preferably, the at least one metabolite is selected from the group consisting of 3HOKYN (3-hydroxykynurenine), AA (anthranilic acid), 3HOAA (3-hydroxyanthranilic acid), KA (kynurenic acid) and QUIN (quinolinic acid).

5        Most preferably, a concentration of each of at least two kynenurine metabolites is measured. Preferably, the at least two kynenurine metabolites are selected from the group consisting of AA and 3HOAA, and KYN and KA.

According to preferred embodiments of the present invention, a ratio of the concentration of the at least two metabolites is measured. Preferably, the  
10        ratio is a ratio of 3HOAA to KYN. Alternatively and preferably, the ratio is a ratio of AA to KYN. More preferably, the ratio is a ratio of the total sum of the concentrations of AA and 3HOAA to the concentration of KYN.

According to other preferred embodiments of the present invention, the concentration of the at least one metabolite is measured by HPLC. Alternatively  
15        and preferably, the concentration of the at least one metabolite is measured by fluorimetry. Also alternatively and preferably, the concentration of the at least one metabolite is measured by an immunochemical assay. More preferably, the immunochemical assay is an ELISA.

According to still other preferred embodiments of the present invention,  
20        the method further comprises the steps of: (d) measuring a concentration of an AED (anti-epileptic drug) in the sample of the subject; and (e) correlating the concentration of the AED with the concentration of the metabolite to determine an efficacy of treatment with the AED. Preferably, the method further

comprises the step of: (f) adjusting a treatment regime for the AED in the subject according to the range of concentrations found in subjects effectively treated with the AED. More preferably, the at least one metabolite is a ratio of the concentrations of KA and 3HOAA, or a ratio of the concentrations of KA and TRP.

According to another embodiment of the present invention, there is provided a method for detecting a predisposition to epilepsy in a subject, the subject being substantially free of the symptoms of clinically detected epilepsy, the method comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least one kynurenine metabolite in the sample; and (c) comparing the concentration of the at least one kynurenine metabolite in the sample to a range of values of the concentration of the at least one kynurenine metabolite for normal individuals, such that if the concentration of the at least one kynurenine metabolite in the sample lies outside of the range of values for normal individuals, the predisposition to epilepsy in the subject is detected.

According to yet another embodiment of the present invention, there is provided a method for determining an efficacy of treatment with an AED (anti-epileptic drug) in a subject, comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least one kynurenine metabolite in the sample; and (c) comparing the concentration of the at least one kynurenine metabolite in the sample to a range of values of the concentration of the at least one kynurenine metabolite for individuals with diagnosed epilepsy

substantially well-controlled by treatment with an AED, such that the efficacy of treatment with the AED in the subject is determined. Preferably, the method further comprises the steps of: (d) measuring a concentration of the AED in the sample of the subject; and (e) correlating the concentration of the AED with the concentration of the metabolite to determine the efficacy of treatment with the AED in the subject.

More preferably, the method further comprises the step of: (f) adjusting a treatment regime for the AED in the subject according to the concentration of the at least one kynurenine metabolite and the concentration of the AED.

According to yet another embodiment of the present invention, there is provided a method for distinguishing between pyridoxine deficiency and pyridoxine dependency in a subject, the method comprising the steps of: (a) obtaining a sample from the subject; (b) measuring a concentration of at least one metabolite formed by kynureninase in the sample; and (c) comparing said concentration of said at least one metabolite formed by kynureninase in the sample to a range of values of said concentration of said at least one metabolite formed by kynureninase for normal individuals, such that if said concentration of said at least one metabolite formed by kynureninase in the sample is higher than said range of values for normal individuals, the presence of pyridoxine dependency in the subject is detected, and such that if alternatively said concentration of at least one metabolite formed by kynureninase in the sample is lower than said range of values for normal individuals, the presence of pyridoxine deficiency in the subject is detected.



According to still another embodiment of the present invention, there is provided a diagnostic system for detecting a predisposition to epilepsy, or epilepsy itself, in a subject, comprising: (a) a sample taken from the subject; and (b) a measurer for measuring a concentration of at least one kynurenine metabolite in the sample.

Preferably, the diagnostic system further includes: (c) a correlator for correlating the concentration of the at least one metabolite in the sample with a range of values for the concentration of the at least one metabolite for normal individuals, such that if the concentration of the at least one metabolite in the sample lies outside of the range of values for normal individuals, the presence of epilepsy in the subject is detected.

According to preferred embodiments of the present invention, the measurer includes a HPLC. Alternatively and preferably, the measurer includes a fluorimeter. Also alternatively and preferably, the measurer includes an immunochemical assay.

The following is a list of abbreviations which are used in the text:

AED	- antiepileptic drugs;
CSF	- cerebro-spinal fluid;
EP	- epilepsy-prone;
ER	- epilepsy-resistant;
TRP	- tryptophan;
KYN	- kynurenine;
KA	- kynurenic acid;

	AA	- anthranilic acid;
	3HOAA	- 3-hydroxyanthranilic acid;
	3HOKYN	- 3-hydroxykynurenine;
	QUIN	- quinolinic acid;
5	3HAO	- 3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6);
	IDO	- indole-amine 2,3-dioxygenase (EC 1.13.11.11);
	GABA	- $\gamma$ -aminobutyric acid;
	NMDA	- N-methyl-D-aspartate;
	PTZ	- pentamethylenetetrazol;
10	PL	- pyridoxal;
	PLP	- pyridoxal 5'phosphate;
	QPRT	- quinolinic acid phosphoribosyltransferase (EC 3.2.2.5);
	KAT	- kynurenine amino-transferase (EC 2.6.1.7);
	PI	- phosphatidylinositol;
15	AP	- alkaline phosphatase (EC 3.1.3.1).

The term "predisposition to epilepsy" includes a predisposition towards seizures without the appearance of any clinical symptoms. Similarly, the term "clinical epilepsy" includes the disease state in which seizures have become clinically detectable. Hereinafter, the term "sample" includes a portion of blood, urine or other body liquid or tissue removed from a subject for the purposes of diagnosis. The term "subject" preferably includes a human who is to be tested, but could also encompass a animal subject to be tested. The term "kynurenine metabolite" or kynurenines includes all tryptophan metabolites formed within

kynurenine pathway of tryptophan degradation up to Niacin: L-kynurenine, 3HOAA, AA, KA or QUIN. Hereinafter the term "anthranilates" includes AA and 3HOAA together.

5    BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 is a graph of a portion of the kynurenine metabolic pathway (the branch for anthranilic acid is not shown);

10    FIGS. 2A-2C and 3A-3B are graphs of the plasma concentrations of TRP, KYN, KA and ratios of KYN/TRP, KA/TRP in EP rats in comparison with ER (Wistar) rats and EP rats are treated with phenytoin;

FIGS. 4 and 5A-5B are graphs of the plasma concentrations of AA and ratios of KA/3HOAA and AA/KYN in EP rats in comparison with ER (Wistar) rats and EP treated with phenytoin;

15

FIGS. 6 and 7 are graphs of the total plasma levels of AA+3HOAA and its ratio to KYN in EP rats in comparison with ER (Wistar) rats and EP treated with phenytoin;

FIGS. 8A and 8B are graphs of plasma concentrations of 3HOAA and its ratio to KYN in EP rats in comparison with ER (Wistar) rats and EP treated with phenytoin;

20

FIGS. 9A and 9B are graphs of the efficacy of phenytoin treatment of sound-induced convulsions (in scores and duration of stages for rats having attacks);

FIGS. 10A and 10B are graphs of the efficacy of constant pyridoxine treatment of sound-induced convulsions (in scores and duration of stages for rats having attacks);

FIGS. 11 - 12 are graphs of the total sum (AA+3HOAA) and its ratio to the total sum ( KYN+ 3HOKYN) in epileptic patients in comparison with healthy individuals;

FIGS. 13 and 14 are graphs of the plasma ratios 3HOAA/KYN and AA/KYN in epileptic patients in comparison with healthy individuals;

FIG. 15 is a graph of the plasma levels of KA in epileptic patients in comparison with healthy individuals; and

FIG. 16 is a graph of the plasma ratios KA/3HOAA in epileptic patients in comparison with healthy individuals.

#### DESCRIPTION OF THE INVENTION

The present invention is of a method for diagnosing clinical epilepsy and of detecting a predisposition to epilepsy, preferably substantially before clinical symptoms become apparent. For diagnosing clinical epilepsy, the method of the present invention is particularly drawn towards clarification of the diagnosis of epilepsy. In addition, other provided embodiments include a method for

optimization of a regimen for AED and diagnostic systems for performing the methods of the present invention.

The method of diagnosing epilepsy or of detecting a predisposition towards epilepsy of the present invention relies upon the altered balance of the concentrations of the metabolites of the kynurenine pathway of tryptophan degradation. This altered balance was found in animal models of genetic epilepsy and in patients with diagnosed epilepsy, both treated and non-treated by AED. Several underdiagnosed cases of epilepsy, as well as of individuals having a predisposition to epilepsy, were detected by using samples taken from the children of epileptic parents. Both clinical and experimental data obtained indicate that the changes in the plasma concentrations of the kynurenine metabolites correlate to the level of seizure predisposition, so that the concentrations of certain kynurenines are biochemical markers indicative of both a predisposition to epilepsy and of developed epilepsy. Under AED treatment some of the biochemical markers of seizure susceptibility are further altered, enabling their measurement to be used for an assessment of the efficacy of treatment.

Also, the concentrations of the kynurenine metabolites L-KYN, AA, 3HOKYN, 3HOAA, KA, xanthurenic acid and QUIN are indicative of the disorders in the absorption, transport or otherwise metabolism of vitamin B6. Without wishing to be bound by a particular mechanism, one possible explanation for this correlation is that an inborn error in vitamin B6-metabolism may form a genetic basis for an enhanced susceptibility to seizures. Regardless

of the particular mechanism, the altered concentrations of the metabolites of PLP-dependent kynurenine pathway can be used as the biochemical markers of the genetic predisposition to epilepsy, as shown in the Examples below.

Furthermore, these parameters are altered during treatment with AED, so that in combination with the measurement of the concentrations of AED in the plasma, these biochemical markers can be used for the effective monitoring of epilepsy therapy by AED.

The background art neither taught nor suggested that such biochemical markers were associated with epilepsy, and certainly did not teach or suggest that these markers could be used to detect a predisposition to genetic seizure disorders and/or the effectiveness of AED treatment in a subject. Furthermore, the background art certainly did not teach or suggest non-invasive methods for a biochemical approach to the diagnosis of epilepsy or of the detection of a predisposition towards epilepsy, such as the analysis of blood plasma, urine or other body liquids and tissues.

Under AED treatment these markers can actually be used for the evaluation of the effectiveness of AED treatment and/or AED overdosing, which was also neither taught nor suggested by the background art.

These methods and diagnostic systems of the present invention could beneficially be used to screen several groups of high-risk individuals. The following groups of individuals at high risk for development of seizure disorders should preferably be tested, including, but not limited to, the first and second degree relatives of patients with epilepsy; babies born through a traumatic or

premature delivery; children with febrile convulsions and attention deficit disorders; patients who have endured brain trauma or intracranial infections; pregnant women; and soldiers and pilots who may be subjected to stressful situations. In addition, epileptic patients treated with AED should preferably be tested, especially those patients who are not being treated effectively.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a method for diagnosing clinical epilepsy, especially for clarification of an existing diagnosis of clinical epilepsy, and of detecting a predisposition to epilepsy, preferably substantially before clinical symptoms become apparent. In addition, other provided embodiments include a method for determining an optimal regime for AED treatment, a method for distinguishing between pyridoxine dependency and pyridoxine deficiency in a subject and diagnostic systems for performing the methods of the present invention.

The method of detecting a predisposition to epilepsy and of clarifying the diagnosis of clinical epilepsy by a biochemical approach according to the present invention relies upon the altered balance of the concentrations of the metabolites of the kynurenine pathway of tryptophan degradation. As further described in the Examples below, the tryptophan metabolites of the kynurenic pathway were examined in the plasma and brain of seizure-naive genetically epilepsy-prone rats and in the plasma of patients with epilepsy; as well as in the plasma of individuals with underdiagnosed epilepsy such as the first and second

degree relatives of affected individuals. These experiments showed that the correlation between the plasma levels of kynurenines and the level of seizure susceptibility existed both in animal models and in clinical materials obtained from epileptic patients. Characteristically increased levels of anthranilates, (AA+3HOAA), the enhanced ratio of anthranilates to their common precursor KYN (AA/KYN, 3-HOAA/KYN), as well as the increased ratio of the total sum of anthranilates to the total sum of both precursors (AA+3-HOAA)/(KYN+3HOAA) were found both in seizure-naive animals genetically predisposed to epilepsy and in epileptic patients. These altered ratios enable the detection of a predisposition to epilepsy and the clarification of the diagnosis of epilepsy according to the methods of the present invention.

In addition, the ratios between the concentration of neuroprotective KA to neurotoxic 3HOAA were compared in AED treated and untreated EP animals, and in AED treated and untreated epileptic patients as well. The data obtained demonstrated that the ratio of KA to 3HOAA and of KA/TRP are especially preferred as the biochemical markers for determining the efficacy of AED treatment and the detection of ineffective or overdosed treatment.

The importance of these markers is emphasized by the results of the animal experiments described below, in which 3-HOAA deficiency was found in certain regions of the epileptic brain. In addition, this animal model was used to study the effects of chronic phenytoin administration and constant pyridoxine treatment on brain kynurenines. Without wishing to be bound by a particular



mechanism, the results obtained suggest that pyridoxine imbalance in the plasma and brain may underlie an increased seizure susceptibility.

#### Example 1

#### 5 Plasma Levels of Kynurenine Metabolites in Seizure-naïve Epilepsy-prone Rats and Epilepsy-resistant Rats

Epilepsy-prone audiogenic sensitive rats (EP) as the animal model of genetic epilepsy were used in comparison with control epilepsy-resistant (ER) Wistar rats. Both strains were developed by, and are available from, Dr. Ch. Marescaux at the Strasbourg UNISERM 398 laboratory.

The plasma levels of the kynurenine metabolites were measured in both groups of rats. Results are given in Figures 2-8. The methods were as follows.

The animals were sacrificed by guillotine. Blood (1.5 ml) was collected in the tubes. After centrifugation (2000 g x10 min at the room temperature) plasma and erythrocytes were kept separately under -80° until processing.

Plasma concentrations of TRP and related metabolites, L-KYN, KA, AA, 3HOKYN and 3HOAA, were measured by the HPLC (High Pressure Liquid Chromatography) method. The reference standards of the metabolites which were measured in the samples (L-TRP, L-KYN, AA, 3HOKYN, 3HOAA and KA, as well as zinc acetate) were purchased from Sigma (USA). Acetonitrile, acetic and perchloric acids were obtained from BioLab (Israel).

Millimolar stock solutions of each standard were prepared and stored at -80°. Blood samples taken from each animal were deproteinized by addition of 100 microliters of 2.4 M perchloric acid to 0.5 ml of plasma. After

centrifugation (10 000 g, 10 min), the supernatants were filtered (0.22 mm filter from Millipore). The samples were then loaded onto the HPLC analyzer.

Specifically, quantitative determinations were obtained using a LaChrom HPLC system (Merck-Hitachi) which includes: LaChrom L-7100 HPLC-Pump, LaChrom L-7200 Autosampler, LaChrom L-7400 UV-VIS Detector , LaChrom L-7480 Fluorescence Detector, LaChrom D-7000 Interface module and LaChrom D-7000 Multi HPLC System Manager Software. The analytical column was a 250 mm x 4 mm I.D., packed with Lichrospher 100 RP-18 (5mm) (Merck, Germany).

100 microliters of each sample were injected into the column. The mobile phase consisted of 50 mM acetic acid, 250 mM zinc acetate (pH 4.9) with 2% (v/v) acetonitrile. The concentration of each metabolite in the sample was determined as the area under the peak, and was corrected by reference to the values obtained for the reference metabolites. Separations were achieved at ambient temperature by isocratic elution at a flow-rate of 1.0 ml/min. The detection and the quantitation were carried out with the fluorescence detector connected on line with a UV Detector. The fluorescence excitation and emission wavelengths were set to 320 nm and 420 nm (respectively) at the beginning of the run. Twenty minutes later, excitation and emission wavelengths were changed to 254 nm and 404 nm. UV signals were then monitored at 365 nm. The concentrations were determined according to the measurement of the peak-area against external standards. The results were as follows.

## Results

The experiments have shown that in the plasma of seizure-naive EP rats the level of KYN and KA are lower, while the levels of both anthranilates, AA and 3HOAA, are higher, than in the plasma of ER Wistar rats.

5       The results presented in Figures 2 and 3 show that in addition to having a significantly higher level of plasma tryptophan, EP animals also have much lower levels of KYN, a KA precursor, and KA itself (see Figures 2B and 3A). Accordingly, lower ratios of KYN to TRP (Figure 2C) and of KA to TRP (Figure 3B) have been found in EP animals as compared to control ER Wistar  
10   rats.

The deficiency of neuroprotective KA becomes even more striking when the level of KA is correlated to the level of neurotoxic 3HOAA, which in EP rats is almost twice higher than in normal rats. The ratio of these functionally opposite metabolites (KA/3HOAA) in epilepsy-prone animals is approximately  
15   38 fold lower than in normal rats (see Figure 4). As an index of seizure susceptibility, this parameter clearly discriminates seizure-naive epilepsy-prone animals and normal ones.

Unlike KA, the concentration of AA, another neuroprotective metabolite (Jhamandas et al, 1990), is more than twice as high in epilepsy-prone rats than  
20   in control ER rats (as shown in Figure 5A), while the ratio of AA to KYN is more than 12 fold higher in EP rats than in ER rats (see Figure 5B).

The total sum of both anthranilates, 3HOAA and AA, is also twice as high in epilepsy-prone rats than in normal ones (Figure 6). The difference

between these results in EP and ER rats becomes especially impressive when the total sum of both anthranilates is correlated to the level of KYN (see Figure 7), and when the level of 3HOAA and AA is separately correlated to the level of KYN, the common substrate of both products (Figure 7B). Given the  
5 increased levels of the anthranilates along with the deficient level of KYN, the value of each of these ratios 3HOAA/KYN, AA/KYN and (AA+3HOAA)/KYN in EP animals is 12-13 times more than in ER rats (see Figures 5,7 and 8A).

The ratio of total sum of both anthranilates (AA+3HOAA) to the total sum of both substrates (KYN +3HOKYN) also gives approximately the same  
10 result, as this ratio is also about twelve time higher in seizure-naive EP animals than in control ER animals.

Thus, these indices calculated for the reactions catalyzed by kynureninase provide evidence for much higher kynureninase activity in genetically EP, but seizure-naive, rats than in normal ER animals. The  
15 constellation of tryptophan metabolites of kynurenine pathway found in the blood plasma provides biochemical markers for distinguishing potentially epileptic animals from normal ones.

Taken together, the lower level of neuroprotective KA and especially the lower ratio of KA to 3HOAA, combined with the increased levels of both  
20 anthranilates and the low concentration of their common precursor KYN, characterize the kynurenine biochemical marker profile for epilepsy predisposition.

Example 2  
Monitoring AED Treatment with Plasma Kynurenines

Since the suggested diagnostic biochemical markers are able to detect  
5 the level of seizure susceptibility as shown in Example 1, their ability to assess  
the efficacy AED treatment was examined. The experiments were carried out in  
the same animal model of genetic epilepsy, the EP and ER rats of Example 1.  
The intensity of sound-induced seizures before and after AED treatment was  
comparatively assessed in the same group of animals and then correlated with  
10 the levels of the kynurenine biochemical markers.

EP audiogenic sensitive rats react to the intensive sound stimulation  
105-120 dB with a running stage ("wild running"), followed by generalized  
tonic-clonic convulsions. Depending on the severity of the reaction, the  
convulsions may end with cataleptic immobility, post-ictal excitation, or coma.  
15 The anticonvulsive effect of phenytoin, the AED which is commonly  
administered clinically for chronic treatment of generalized tonic-clonic  
convulsions, was shown to be correlated with the biochemical markers of  
seizure susceptibility of the present invention.

Eleven EP audiogenic sensitive rats and eleven control ER rats were used  
20 in experiments at the age of 2.5 months. When subjected to the audiogenic  
stimulation (110 db, 30 sec), all 11 audiogenic-sensitive EP rats developed  
severe generalized tonic-clonic convulsions, ended by coma (in 6 animals), or  
post-ictal excitation (5 rats). Estimated in scores, the intensity of audiogenic

convulsions in this group of animals was 10.5 (see Figures 9 and 10). The scores were estimated as follows:

- 0 - absence of reactions
- 1 - short-term running stage
- 5 2 - two-phase running stage
- 4 - tonic convulsions of flexors
- 6 - tonic convulsions of extensors
- 8 - postictal excitation or muscle atonia
- 10 - postictal excitation with convulsions
- 10 12 - postictal coma
- 16 - death

The mean duration of the stages of audiogenic attacks is shown in Figure 9B. No sound-induced convulsive reactions were observed in the control group.

Starting the following day, the EP animals were injected with phenytoin.

- 15 The treatment protocol included a first injection of phenytoin 75 mg/kg i.p., followed by 12 successive injections of phenytoin (50 mg/kg) once per day (Loscher et al., 1985).

After 13 days of phenytoin treatment, the incidence and intensity of sound-induced convulsions were estimated again. Each animal was tested on day 13 and again on day 14. Altogether 22 tests of sound stimulation were carried out. The patterns of the audiogenic reactions observed before and after chronic phenytoin treatment were compared (see Figure 9). Phenytoin administered according to the above protocol provided a protective effect

against sound-induced generalized tonic-clonic convulsions. Sixteen out of the twenty-two tests of sound stimulation did not induce any convulsions, so that only six tests resulted in sound-induced convulsions. The intensity of these six sound-induced convulsive reactions was significantly reduced: the duration of tonic convulsions (the most severe stage of the attack) was remarkably shorter, while the duration of the latency and running stage phases (the least traumatic stages of a sound-induced convulsive attack) were longer, than in non-treated EP rats. There were neither post-ictal excitation, nor coma. Furthermore, cataleptic immobility arose at the moment when the sound ceased, or even preceded this moment (see Figure 9).

The next day, eight animals were selected which did not convulsively react to the sound stimulation when repeated twice, showing that these EP rats were completely protected by chronic phenytoin treatment. These eight selected animals were sacrificed, and blood samples were taken, processed and analyzed as described above for Example 1.

A significant decrease in the level of TRP with an appropriate increase in the levels of KYN, and especially of neuroprotective KA, characterise the effect of the phenytoin treatment in EP animals. The level of KA in phenytoin treated EP animals turned out to be 13 fold higher than in non-treated EP rats and 1.43 fold higher than in the control ER rats (370 nM vs. 260 nM; see Figure 3). Also, the ratio of KA to TRP in phenytoin treated animals was strongly higher than in the control ER rats. In addition, the concentration of neurotoxic 3OHAA in phenytoin-treated rats was a bit lower than in non-treated EP rats and almost

equal to the level found in control ER rats (see Figure 8B). The opposing trends of the concentrations of these two functionally opposite metabolites results in a dramatic increase in the mean value of KA/3OHAA ratio under phenytoin treatment. This ratio in phenytoin treated EP rats was about 35 fold higher than in non-treated epilepsy-prone rats, and was equal to the mean value found in the control ER Wistar rats (see Figure 4).

Thus, the plasma ratio of KA/3-HOAA correlates with the level of seizure susceptibility under AED treatment. The increased value of this ratio corresponds to the lower level of seizure predisposition achieved from the regular administration of the AED phenytoin.

Moreover, AED overdosing can be diagnosed by the suggested marker, since a striking excess of both KA and of the ratios of KA/TRP and KA/3-HOAA in phenytoin-treated epileptic animals was found to be indicative for phenytoin overdose during the course of treatment. For example, the observed concentrations of KA at 875.7 nM and 1341.4 nM produced corresponding KA/3-HOAA ratios equal to 101.8 and 72.1 respectively, which were significantly higher than the equivalent measurements characteristic of control ER Wistar rats, and indicated phenytoin overdosing. By contrast, concentrations of 222.6 nM and 223.5 nM KA produced corresponding KA/3-HOAA ratios equal to 15.9 and 15.5 respectively, were at the end of the range of values observed for normal animals and indicated that the individual dose of AED could be increased.



Some other observed symptoms, such as hair loss and impaired motor coordination, also pointed to phenytoin overdosing. Hard (stone-like) liver, regional bleeding in the lungs, and enlarged adrenal glands were found when the animals were sacrificed and also corroborated the hypothesis of a toxic overdose of phenytoin (data not shown).

The measurement of the KA level and both the ratios of KA/3HOAA and KA/TRP, along with the concentrations of AED in plasma which are currently measured, should improve the monitoring of AED treatment in epileptic patients.

10

### Example 3 Analysis of Brain Kynurenines in Epilepsy-prone Rats

The analysis of the levels of kynurenines in the brain was performed in the same animal model of genetic epilepsy, seizure-naive EP audiogenic sensitive rats, in comparison with ER Wistar rats, as in Example 1 above. In addition, the level of brain kynurenines was also determined in EP rats chronically treated with phenytoin (see Example 2 above), and in the separate group of seizure-naive EP rats constantly treated with pyridoxine (75 mg/L).

### Methods

Four groups of animals were used for the comparative analysis of brain kynurenines: (1) seizure-naive EP rats; (2) seizure-naive EP rats constantly treated by pyridoxine; (3) EP rats chronically treated with phenytoin according to the protocol described in Example 2; and (4) control ER Wistar rats. As in Example 2, only those animals which were completely protected by phenytoin

treatment against sound-induced convulsions were used for the analysis of brain kynurenines.

Pyridoxine treatment. The administration of pyridoxine was started on day 3 after birth, when half of 4 litters of EP rats (18 offspring) were given pyridoxine in the drinking water (75 mg/L). For the first 3 weeks pyridoxine was administered to the lactating mothers, after which it was directly administered to the offspring themselves constantly until the animals were sacrificed. Seven of these pyridoxine-treated animals were used for detection of kynurenines in brain tissue, while others were tested for sensitivity to the sound-induced convulsions in comparison with untreated EP rats of the same litters.

The animals were sacrificed at the age of 2.5 months. Brain tissue was dissected on the cold plate. The samples of the cortex, brain stem, midbrain and cerebellum were collected and immediately frozen for storage at -80°C. The homogenized brain samples were prepared for HPLC detection and analysed as it was described above.

## Results

The experiments showed that the intensity of the sound-induced convulsions (estimated in scores) was much lower in pyridoxine-treated animals than in untreated EP animals (see Figure 10). The duration of tonic convulsions, which is the most severe stage of the reaction, was also reduced, while the duration of less severe stages of the reaction, including latency, running stage ("wild running") and clonic seizures was longer than in untreated EP rats. Post-

ictal conditions were also less severe in pyridoxine-treated animals than in untreated EP rats, since only post-ictal immobility was demonstrated while post-ictal coma or excitation predominated in the untreated EP group.

As seen in Table 1, the concentrations of kynurenines in the brain is  
5 significantly different from the concentrations of these metabolites in the plasma. Without wishing to be bound by a single mechanism, the restricted brain-blood barrier transport for some of the kynurenines, along with altered activity of the enzymes of kynurenic pathway in the brain, may cause the difference between the concentrations of kynurenines in the plasma and brain  
10 (Fukui et al., 1991; Stone, 1993; Luthman et al., 1996) . Moreover, the profile of TRP metabolites of the kynurenic pathway is rather specific in each area of the brain which was examined.

Only TRP and 3HOAA were regularly detected in all brain regions studied both in ER Wistar and in EP animals, while the amounts of other  
15 compounds, including KYN, 3-HOKYN, AA and KA varied from non-detectable up to significant concentrations.

The compound AA was found in practically equal concentrations in the cortex, midbrain and the brain stem of EP rats (see Table 1). The relative ease of penetration of AA through the brain-blood barrier ( Fukui et al., 1991) along  
20 with the highly increased level of AA in the plasma of EP animals may enable a significant amount of AA of peripheral origin to enter the brain pool of AA in EP animals.

The concentrations of 3HOAA in the cerebellum, the main inhibitory brain structure, were significantly different in EP and ER animals. The level of 3HOAA in the cerebellum of EP animals was 3 fold lower than in the cerebellum of ER Wistar rats (see Table 1). Although the total concentration of 3HOAA in the brain stem of EP rats was 30% lower than in ER rats, the *relative concentration* of 3HOAA to the concentration of TRP in the brain stem was equal in EP and ER animals. The comparison of the relative concentrations of 3HOAA in the cerebellum of EP and ER animals confirms the significant deficiency in the production of 3HOAA found in the cerebellum of EP rats. A decrease in the relative concentration of 3HOAA has also been found in the midbrain, the region where the focus of epileptic activity arises in sound-induced seizures of EP rats.

Unlike other brain structures which were examined, about 40% of the excess 3-HOAA and the corresponding increase in the 3-HOAA/TRP ratio was found in the cortex of EP rats in comparison with ER animals. Without wishing to be limited by a single mechanism, such a significant feature of the kynurenine metabolism in the cortex should be considered in combination with other distinctive features of the cortical metabolism found in EP animals, such as serotonin deficiency which had only been found in the cortex of EP mice; the increase in the ratio of glutamate to GABA which, unlike other brain regions, was not corrected by constant pyridoxine administration; and unlike other brain regions, the equal intensity of PI turnover in EP and ER mice (Dolina and Kozak, 1987; Vriend et al., 1991; Dolina et al., 1993).

Since 3HOAA poorly crosses the brain-blood barrier, a lower rate of transformation of 3HOKYN into 3HOAA may be explained by the lower activity of kynureninase in the cerebellum of EP animals. The low activity of kynureninase in this structure is also confirmed by an absence of AA in the cerebellum of EP animals, unlike other brain structures of these animals.

The restoration of the cerebellar 3HOAA level, up to the level characteristic of control ER Wistar rats, is the common outcome of therapy with both phenytoin and pyridoxine, at least in terms of their influence on the metabolism of kynurenines.

Without wishing to be bound by a single mechanism, the biochemical correction of the cerebellar inhibitory function, together with an increase in the level of neuroprotective KA in the brain stem, may contribute to the inhibition of audiogenic convulsions provided by phenytoin. The mitigation of audiogenic seizures under pyridoxine treatment is probably achieved by an increase in the cortical level of KA, in addition to the correction of the concentration of 3-HOAA in the cerebellum.

Reduced 3HOAA concentration and the value of the 3HOAA/TRP ratio in the cerebellum of EP animals, together with the same tendency in the midbrain and the brain stem, may point to the altered activity of the kynurenine pathway enzymes in these brain regions in EP animals. As noted previously, kynureninase is highly sensitive to PLP supply (Bender, 1989). Kynureninase forms AA from KYN and 3HOAA from 3HOKYN. Therefore, PLP

insufficiency should be one of the main factors affecting kynureninase activity in these regions of the brain of EP animals.

Pyridoxine is known to readily enter CSF and the brain from plasma by a saturable transport mechanism (Spector, 1978). The major transport form of vitamin B6 is pyridoxal (PL). PL easily crosses membranes and is the form in which vitamin B6 is taken up into a cell (Lumeng & Li, 1985). Hydrolysis of extracellular PLP by membrane-bound alkaline phosphatase (AP) initiates the vitamin B6 absorption by a cell. Thus, AP is critical for regulation of the cellular content of PLP (White et al. 1985, 1988; Lumeng 1986), and a deficiency in the activity of AP may disrupt the intracellular uptake of vitamin B6, as well as the transport of vitamin B6 from one cell compartment to another.

Recently, the critical role of AP in vitamin B6 metabolism and seizure development found a strong confirmation from animal studies. Waymire et al.(1995) have shown that mutant mice lacking a functional AP gene develop seizures which are subsequently fatal. Defective metabolism of PLP in these animals and specifically the elevation of serum PLP level, was shown. Animals displaying the mutant seizure phenotype can be rescued by the administration of PL.

Without wishing to be bound by a single mechanism, these data support the hypothesis that a type of primary AP deficiency may be the cause of both interrelated disorders found in epilepsy from the experiments detailed in the previous Examples: 1) PLP excess in the plasma and 2) a reduction of vitamin B6 transport into some brain regions. The first disorder, among other effects,

results in an over-excess of the anthranilates (kynureninase products) and an elevation of 3HOAA/KYN, AA/KYN and (3HOAA+AA)/KYN values in the plasma; the second results in a decrease in kynureninase activity, and consequently, in a reduction of 3HOAA synthesis in the specific brain regions.

5        AP is known to be a phosphatidylinositol (PI)-anchored protein, which is released into the serum in a variety of pathological and physiological situations, causing an anchor-degrading effect. In our previous experiments (Dolina, Kozak, 1987) an intensified turnover of (PI) was found in the brain stem and cerebellar plasmatic membranes of seizure-naïve EP BALB/c mice, in  
10       comparison with ER animals of the same strain. Subconvulsive PTZ-loading resulted in the intensive degradation of PI in EP animals, in contrast to PI accumulation in ER animals. Hence, these data obtained in another animal model of genetic epilepsy support the hypothesis of AP deficiency in epilepsy.

      The effects of phenytoin, as well as of pyridoxine itself, also corroborate  
15       this claim. An increase in the levels of 3-HOAA and 3HOAA/TRP in the cerebellum of EP rats in the course of chronic anticonvulsive therapy with both compounds seems to be - in terms of brain kynurenines - one of the main effects of the compounds. Phenytoin is well known to induce the elevation of serum AP during long-term anticonvulsive therapy (Kutt, Lowis 1970; Kazamatsuri 1970;  
20       Lefebvre et al 1972; Tjellesen et al 1983). AP activation should result in an increase in vitamin B6 transport into the brain, providing among other effects a restoration of kynureninase activity and an increase of the 3HOAA levels in the specific brain regions. The biochemical correction of the cerebellar inhibitory

function, together with an increase in the level of neuroprotective KA in the brain stem, contributes to the inhibition of audiogenic convulsions provided by phenytoin. The mitigation of audiogenic seizures under pyridoxine treatment is probably achieved - at least partially - by an increase in the cortical level of KA.

5 Hence, the difference in concentrations of 3HOAA in some of brain regions of EP and control ER animals, and anticonvulsive effects of phenytoin and pyridoxine as well, indicate the key role of vitamin B6 imbalance in the development of predisposition to epilepsy.

10

#### Example 4 Tryptophan Metabolites in Plasma as Biomarkers of Clinical Epilepsy

The diagnostic markers developed in the animal model of genetic epilepsy were examined to determine their clinical applications. In particular,  
15 the levels of these markers were measured in both healthy and epileptic human subjects to determine if epileptic patients can be distinguished from the normal population with these markers. In addition, the possible correlation of the levels of these markers to the extent of seizure predisposition in epileptic patients was examined. Finally, the alteration of the value of the markers by AED treatment  
20 was examined.

#### Method

The measurement of kynurenines in human blood samples was performed as described above for Examples 1 and 2.



## Results

The data presented in Table 2 indicate that with equal concentrations of TRP, the plasma concentrations of KYN, 3HOAA, AA and KA in epileptic patients are significantly different from healthy individuals. In the control group the concentrations of these compounds were close to the scanty data presented in the literature as the normal ranges for tryptophan metabolites (Geigy Scientific Tables, 1984). In epileptic patients, both treated and untreated with AED, the concentrations of these compounds strongly differed from the levels observed in healthy individuals.

The distinctions between the groups were dramatic when the levels of AA and 3HOAA, and especially of the total sum of both anthranilates (AA+3HOAA) characteristic of each group were compared. In epileptic patients, the plasma level of AA+3HOAA was about 20 times higher than in the healthy group (see Figure 11). AED treatment did not abolish this difference, since the total sum of anthranilates was almost the same in the treated and non-treated epileptic patients. If the levels of both anthranilates (AA+3HOAA) were correlated to the levels of both precursors (KYN+3HOKYN) in the reactions catalysed by kynureninase, the difference between the normal individuals and epileptic patients remained striking (see Figure 12). Even much higher distinctions between the groups are found when the level of 3HOAA, an immediate precursor of QUIN which mimics its neurotoxic effect, was correlated to the level of KYN (see Figure 13). This latter ratio was

approximately 50 fold higher in non-treated epileptic patients than in healthy individuals. Even if the single largest value of the ratio (2425) obtained in one of the cases is excluded, a 12 fold difference between the groups still remains.

With regard to monitoring AED treatment, a significant decrease in the  
5 level of 3HOAA, along with an increase in the level of KYN, resulted in a normalization of 3HOAA/KYN ratio with effective AED treatment (see Figure 13). In contrast to the 3HOAA/KYN index, the ratio of AA to KYN, which was also found to be highly different between the healthy individuals and epileptic patients, was not corrected under AED treatment (see Figure 14). This  
10 difference may reflect much stronger sensitivity to the effect of AED treatment of the kynureninase-catalyzed conversion of 3HOKYN to 3HOAA than of the conversion of KYN to AA.

As shown in Tables 2-4, the concentrations of KYN was higher in both groups of epileptic patients than in the control group. The concentration of  
15 neuroprotective KA was also higher in epileptic patients than in the healthy group. In the group of epileptic patients whose epilepsy was successfully controlled by AED drugs, the concentration of KA was strongly higher than in the control group. In the non-treated group of epileptic patients, the KA level was almost equal to the level of KA in the healthy individuals (see Figure 15).  
20 The highest concentration of neurotoxic 3HOAA was observed in the non-treated group of patients with epilepsy ( $84.5 \pm 27.9$  nM/ml); in the AED treated group, the concentration of this compound was 3 times higher than in healthy individuals ( $8.68 \pm 3.2$  as opposed to  $3.45 \pm 1.79$ ). Accordingly, the

KA/3HOAA ratio, a marker of seizure predisposition, was two fold lower in non-treated epileptic patients than in healthy individuals, and about 7 fold lower than in patients whose epilepsy was controlled by AED (see Figure 16).

Thus, the peculiar constellation of alterations to kynurenine metabolites, namely the highly increased total concentration of anthranilates (AA + 3HOAA) and the ratio 3HOAA/KYN, along with a highly diminished KA/3HOAA ratio, specifically differentiated epileptic patients not being treated with AED from healthy individuals.

Unlike untreated epileptic patients, the value of 3HOAA/KYN was within the normal range for AED treated patients (Figure 13), and the value of KA/3HOAA was increased up to the level characteristic of healthy individuals. The two last markers may be suitable for clinical monitoring of AED treatment.

Hence, the markers of the 3HOAA/KYN ratio and the KA/3HOAA ratio, shown in the previous Examples to be indicative of the efficacy of AED treatment in the animal model of genetic epilepsy, were also shown to be effective for the monitoring of AED treatment in epileptic patients (see Figures 4 and 16, and Figures 8 and 13).

Example 5  
Methods and Diagnostic Systems for  
the Diagnosis of Epilepsy and for  
the Detection of a Predisposition to Epilepsy

From the biochemical markers found and evaluated in Examples 1-4 above, methods and diagnostic systems for the diagnosis of both clinical and pre-clinical epilepsy can be made. In particular, the diagnosis of clinical

epilepsy could be clarified with the methods and diagnostic systems of the present invention. A predisposition for epilepsy could also be detected.

The methods would involve the measurement of at least one kynurenine metabolite in the sample taken from the subject. In one embodiment, the method involves the following steps. First, the sample is obtained from the subject. Preferably, the sample is a blood sample, which could be withdrawn with a needle, for example, according to well known procedures in the art. Alternatively and preferably, the sample is a urine sample, although any other fluid or tissue sample could be used. Next, the level of at least one kynurenine metabolite in the sample would be measured. Preferably, the metabolite is selected from the group consisting of KYN, 3HOAA, 3HOKYN, AA and KA. More preferably, the concentrations of two metabolites are measured, either the anthranilates AA and 3HOAA or KYN and 3HOKYN. Most preferably, a ratio of the concentrations of at least two metabolites is measured, such as the ratio of the concentration of 3HOAA to the concentration of KYN ( $3HOAA/KYN$ ), the ratio of the concentration of AA to the concentration of KYN ( $AA/KYN$ ), or the ratio of the total concentrations of AA and 3HOAA ( $AA+3HOAA$ ) to the concentration of KYN ( $(AA+3HOAA)/KYN$ ).

As described in further detail below, the level could be measured through HPLC, fluorimetry or an immunological test such as an ELISA, for example. The measured level would then be compared to the range of values for normal individuals without epilepsy. As described above, the expected results of the plasma level of AA + 3HOAA should be approximately 20 times higher, in

epileptic patients than in the healthy group. Furthermore, all three ratios, the ratio of the concentration of 3HOAA to the concentration of KYN, the ratio of the concentration of AA to the concentration of KYN, and the ratio of the total concentrations of AA and 3HOAA to the concentration of KYN, should be  
5 greater in epileptic subjects than in normal subjects.

According to another embodiment, a similar method could be used to distinguish between pyridoxine dependency and pyridoxine deficiency conditions in the subject. Pyridoxine deficiency may be found in elderly patients or patients with chronic alcoholism or malnutrition, and is defined as a  
10 reduction in the amount of vitamin B6 in the patient. Pyridoxine dependency, however, is found in patients with epilepsy and a predisposition to epilepsy, and may be related to a disordered transport of vitamin B6 within tissues and the resultant increase in the blood level of vitamin B6. Distinguishing between these two conditions could be very important for the proper treatment of the  
15 patient.

These two conditions can be distinguished by obtaining a sample from the subject, and then by measuring the level of at least one kynurenine metabolite in the sample. The level is then compared to a range of values for the concentration of that metabolite in normal individuals. If the subject has  
20 pyridoxine dependency, the concentration of the metabolites formed by kynureninase, such as AA and 3HOAA, in the sample should be higher than the range of values for these metabolites in normal individuals. On the other hand, if the subject has pyridoxine deficiency, the concentration of AA and/or

3HOAA in the sample should be lower than the range of values for normal individuals. Thus, the comparison is preferably made with at least one metabolite formed by kynureninase, such as AA and/or 3HOAA.

According to still another embodiment, the method of the present invention could be used to determine the efficacy of AED treatment. First, the sample would be obtained from the subject as previously described. Next, the level of at least one kynenurine metabolite would be measured. Preferably, the metabolite would be selected from the group consisting of KA, 3HOAA and TRP. More preferably, a ratio of the concentrations of at least two metabolites, such as the ratio of the concentration of KA to the concentration of 3HOAA or the ratio of the concentration of KA to the concentration of TRP, would be measured. Next, the level of at least one metabolite or the ratio would be preferably compared to the range of values obtained for normal subjects, and optionally to the range of values obtained for subjects with non-controlled epilepsy and subjects with well-controlled epilepsy, in order to assign the tested subject to one of these three groups and hence to make the diagnosis of successfully controlled or non-successfully controlled epilepsy.

As noted above, successful treatment of epilepsy with AED should result in the normalization of the 3HOAA/KYN ratio. According to the statistical data obtained, the KA/3HOAA ratio is about two fold lower in non-treated epileptic patients than in healthy individuals, and about 7 fold lower than in patients whose epilepsy was well-controlled by AED. For individual metabolites, the concentration of KA should be higher in subjects with successfully controlled

epilepsy than in normal individuals. The KA level in the non-treated group of epileptic patients is only slightly higher than in the healthy individuals. The highest concentration of neurotoxic 3HOAA is found in the non-treated group of patients with epilepsy, while in the AED treated group, the concentration of this compound should still be almost 3 times higher than in healthy individuals.

Preferably, each of these measurements of at least one kynenurine metabolite is correlated with a measurement of an AED level in a sample of the subject, more preferably the same sample of the subject. This correlation could be used to determine whether the amount of AED being administered should be altered by being increased or decreased, for example, or whether a different AED should be administered.

Another embodiment of the present invention would be a diagnostic system for implementing the methods of the present invention. The system would include a measurer for measuring a concentration of at least one kynurenine metabolite in a sample taken from a subject, substantially as described for the methods above. The system would also preferably include a correlator for correlating the measured concentration with a range of values of this concentration observed in normal subjects. The correlator could be a software program written in a computer language such as C or C++ and operated by a computer, for example. One of ordinary skill in the art could easily construct such a software program.

A number of different technical approaches to the measurer of the diagnostic system are possible. As described in the previous Examples, the first

approach is to use HPLC to analyze the blood samples or other samples taken from patients. However, this approach can be technically difficult to implement on a wide scale. Therefore, one of three other approaches could also be used. It should be noted that these are intended as examples only and are not meant to be limiting.

Spectrofluorimetric measurement of anthranilates ( AA+3HOAA) and KA in the same sample

The difference in the spectra of fluorescence of anthranilates and kynurenic acid permits discrimination between them in the same sample, even if HPLC is not used to separate these metabolites. While for anthranilates the optimal excitation and emission wavelengths are at 320 nm and at 420 nm (respectively), these parameters for KA are 254 and 404 nm (respectively). Furthermore, both AA and KA fluoresce only in the presence of an additional molecule, zinc salts in these experiments, while the other metabolites analyzed in the same sample using HPLC with a fluorimetric detector either do not fluoresce (such as L-KYN and 3-hydroxykynurenine), or do not fluoresce without the additional molecule.

Therefore, the measurements of the parameters of fluorescence in the presence or absence of the specific helper molecule provide the possibility of separately detecting the total concentrations of anthranilates (AA+ 3HOAA) and KA.



### Activity of key enzymes of kynurenine pathway.

The concentrations of metabolites depend on the activity of the key enzymes of the metabolic pathway. The rate limiting enzymes of the kynurenine pathway are kynureninase, kynurenine-3-hydroxylase, 3-hydroxyanthranilic acid-dioxygenase and QUIN phosphoribosyl-transferase (QPRT).

Some of these enzymes are found in the blood cells: kynureninase activity has been detected in lymphocytes, while QPRT activity was found in erythrocytes. Therefore, homogenates of lymphocytes can be used for further detection of the activity of kynureninase, while homogenates of erythrocytes can be used for detection of QPRT activity. In addition, the activity of alkaline phosphatase, which as previously described may be involved in the defect in the metabolism of kynurenines, can also be measured in peripheral tissues such as blood cells.

### Immunochemical Detection

Immunochemical detection is based on a highly specific antigen-antibody reaction. Such detection is highly effective and reliable in operation. Several steps should be performed to develop suitable assays for immunochemical detection. First, an antigen must be developed. Since the kynurenines are low molecular weight compounds, they should be linked as haptens to compounds of high molecular weight (such as a protein, polysaccharide, or a synthetic polymer) to prepare "anti-kynurenine" antibodies, as is well known in the art. For example, the kynurenines could be linked to keyhole limpet hemocyanin as a protein carrier for immunization. Next, animals must be immunized to

develop highly specific antisera. Optimal antisera must then be selected.

Finally, the conditions for the reaction must be optimized, including time,

temperature, concentrations of reagents and the method to detect the antigen-

antibody (such as ELISA). Both polyclonal and monoclonal antibodies can be

5 used for the development of these assays, as can other immunologically reactive molecules such as Fab' fragments.

Once the assay has been developed, the evaluation of the effectiveness of the assay for the pre-clinical assessment of seizure predisposition is performed.

The blood samples are compared to the samples taken from control non-

10 epileptic patients.

It will be appreciated that the above descriptions are intended only to serve as examples, and that many other embodiments are possible within the spirit and the scope of the present invention.

## WHAT IS CLAIMED IS:

1. A method for diagnosing epilepsy in a subject, the method comprising the steps of:
  - (a) obtaining a sample from the subject;
  - (b) measuring a concentration of at least one kynurenine metabolite in the sample; and
  - (c) comparing said concentration of said at least one kynurenine metabolite in the sample to a range of values of said concentration of said at least one kynurenine metabolite for normal individuals, such that if said concentration of said at least one kynurenine metabolite in the sample lies outside of said range of values for normal individuals, the diagnosis of epilepsy in the subject.
2. The method of claim 1, wherein the sample is a blood sample.
3. The method of claim 1, wherein the sample is a urine sample.
4. The method of claim 1, wherein said at least one metabolite is selected from the group consisting of KYN (kynurenine), 3HOKYN (3-hydroxykynurenine), AA (anthranilic acid), 3HOAA (3-hydroxyanthranilic acid), KA (kynurenic acid) and QUIN (quinolinic acid).

5. The method of claim 4, wherein said at least one metabolite is selected from the group consisting of 3HOKYN (3-hydroxykynurenine), AA (anthranilic acid), 3HOAA (3-hydroxyanthranilic acid), KA (kynurenic acid) and QUIN (quinolinic acid).

6. The method of claim 4, wherein a concentration of each of at least two kynenurine metabolites is measured.

7. The method of claim 6, wherein said at least two kynenurine metabolites are selected from the group consisting of AA and 3HOAA, and KYN and 3HOKYN.

8. The method of claim 6, wherein a ratio of said concentrations of said at least two metabolites is measured.

9. The method of claim 8, wherein said ratio is a ratio of said concentration of 3HOAA to said concentration of KYN.

10. The method of claim 8, wherein said ratio is a ratio of said concentration of AA to said concentration of KYN.

11. The method of claim 8, wherein said ratio is a ratio of said total concentrations of AA and 3HOAA to said concentration of KYN.

12. The method of claim 6, wherein a concentration of each of substantially all of said kynurenine metabolites is measured.
13. The method of claim 4, wherein said concentration of said at least one metabolite is measured by HPLC.
14. The method of claim 4, wherein said concentration of said at least one metabolite is measured by fluorimetry.
15. The method of claim 4, wherein said concentration of said at least one metabolite is measured by an immunochemical assay.
16. The method of claim 15, wherein said immunochemical assay is an ELISA.
17. The method of claim 1, further comprising the steps of:
  - (d) measuring a concentration of an AED (anti-epileptic drug) in the sample of the subject; and
  - (e) correlating said concentration of said AED with said concentration of said metabolite to determine an efficacy of treatment with said AED.
18. The method of claim 17, further comprising the step of:

- (f) adjusting a treatment regimen for said AED in the subject according to said efficacy of treatment with said AED.

19. A method for detecting a predisposition to epilepsy in a subject, the subject being substantially free of signs and symptoms of clinical epilepsy, the method comprising the steps of:

- (a) obtaining a sample from the subject;
- (b) measuring a concentration of at least one kynurenine metabolite in the sample; and
- (c) comparing said concentration of said at least one kynurenine metabolite in the sample to a range of values of said concentration of said at least one kynurenine metabolite for normal individuals, such that if said concentration of said at least one kynurenine metabolite in the sample lies outside of said range of values for normal individuals, the predisposition to epilepsy in the subject is detected.

20. A method for determining an efficacy of treatment with an AED (anti-epileptic drug) in a subject, comprising the steps of:

- (a) obtaining a sample from the subject;
- (b) measuring a concentration of at least one kynurenine metabolite in the sample; and

- (c) comparing said concentration to an expected range of values for individuals with diagnosed epilepsy substantially controlled by treatment with an AED, such that the efficacy of treatment with the AED in the subject is determined.

21. The method of claim 20, further comprising the steps of:

- (d) measuring a concentration of the AED in the sample of the subject; and
- (e) correlating said concentration of the AED with said concentration of said metabolite to determine the efficacy of treatment with the AED in the subject.

22. The method of claim 21, further comprising the step of:

- (f) adjusting a treatment regimen for the AED in the subject according to the efficacy of treatment with the AED.

23. A diagnostic system for detecting a presence of epilepsy in a subject, comprising:

- (a) a sample taken from the subject; and
- (b) a measurer for measuring a concentration of at least one kynurenine metabolite in said sample.

24. The diagnostic system of claim 23, further comprising:

- (c) a correlator for correlating said concentration of said at least one metabolite in said sample with a range of values for said concentration of said at least one metabolite for normal individuals, such that if said concentration of said at least one metabolite in said sample lies outside of said range of values for normal individuals, the presence of epilepsy in the subject is detected.

25. The diagnostic system of claim 24, wherein the sample is a blood sample.

26. The diagnostic system of claim 24, wherein the sample is a urine sample.

27. The diagnostic system of claim 24, wherein said at least one metabolite is selected from the group consisting of KYN (kynurenine), 3HOKYN (3-hydroxykynurenine), AA (anthranilic acid), 3HOAA (3-hydroxyanthranilic acid), KA (kynurenic acid) and QUIN (quinolinic acid).

28. The diagnostic system of claim 27, wherein a concentration of each of at least two kynenurine metabolites is measured by said measurer.



29. The diagnostic system of claim 28, wherein said at least two kynurenine metabolites are selected from the group consisting of AA and 3HOAA, and KYN and 3HOKYN.

30. The diagnostic system of claim 29, wherein a ratio of said concentrations of said at least two metabolites is measured by said measurer.

31. The diagnostic system of claim 30, wherein said ratio is a ratio of said concentration of 3HOAA to said concentration of KYN.

32. The diagnostic system of claim 30, wherein said ratio is a ratio of said concentration of AA to said concentration of KYN.

33. The diagnostic system of claim 30, wherein said ratio is a ratio of a total of said concentrations of AA and 3HOAA to said concentration of KYN.

34. The diagnostic system of claim 24, wherein said measurer includes a HPLC.


35. The diagnostic system of claim 24, wherein said measurer includes a fluorimeter.

36. The diagnostic system of claim 24, wherein said measurer includes an immunochemical assay.

37. The diagnostic system of claim 36, wherein said immunochemical assay is an ELISA.

38. A method for distinguishing between pyridoxine deficiency and pyridoxine dependency in a subject, the method comprising the steps of:

- (a) obtaining a sample from the subject;
- (b) measuring a concentration of at least one metabolite formed by kynureninase in the sample; and
- (c) comparing said concentration of said at least one metabolite formed by kynureninase in the sample to a range of values of said concentration of said at least one metabolite formed by kynureninase for normal individuals, such that if said concentration of said at least one metabolite formed by kynureninase in the sample is higher than said range of values for normal individuals, pyridoxine dependency in the subject is detected, and such that if alternatively said concentration of at least one metabolite formed by kynureninase in the sample is lower than said range of values for normal individuals, pyridoxine deficiency in the subject is detected.

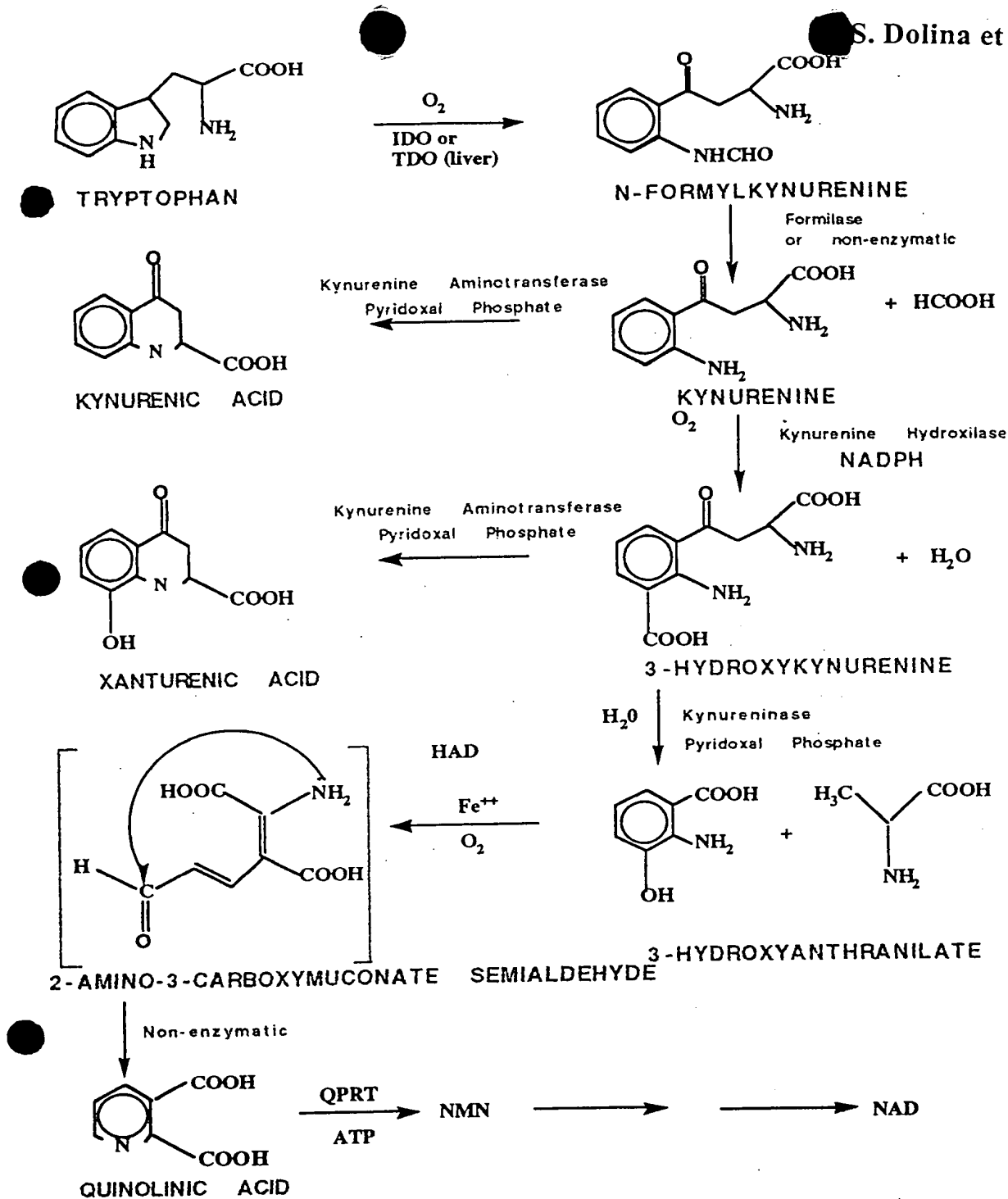
  
**DR. MARK FRIEDMAN LTD.**  
**PATENT ATTORNEYS**  
**SAMUELOFF BUILDING**  
**7 HAOMANIM STREET**  
**67897 TEL-AVIV ISRAEL**

## APPENDIX - REFERENCES

1. Andermann E. Multifactorial inheritance of generalized and focal epilepsy. In: Anderson VE, Hauser WA, Penry JK, Sing CF, ed. *Genetic Basis of the Epilepsies*. New York: Raven Press 1982:355-74.
2. Deldago-Escueta AV, Treiman DM, Enrile-Bascal F. Phenotypic variations of seizures in adolescent and adults. In: *Genetic Basis of the Epilepsies*. New York: Raven Press 1982:49-81.
3. Dolina S, Kozak H. Is B6 dependency an essential factor of genetic predisposition to epilepsy? In: *17-th Epilepsy International Congress Book of Abstracts*, Jerusalem 1987:34.
4. Dolina S, Peeling J, Sutherland G, Pillay N, Greenberg A. Effect of sustained pyridoxine treatment on seizure susceptibility and regional brain amino acid levels in genetically epilepsy-prone BALB/c mice. *Epilepsia* 1993; 34:33-42.
5. French JH, Grueter BB, Druckman R, O'Brien D. Pyridoxine and infantile myoclonic seizures. *Neurology* 1965; 15,2:101-112.
6. Fukui S, Schwarcz R, Rapoport SI, Takada Y, Smith QR. Blood-brain barrier transport of kynurenines: implication for brain synthesis and metabolism *J. Neurochem* 1991; 56: 2007-17.
7. Heyes MP, Saito K, Devinsky O, Nadi NS. Kynurenine pathway metabolites in cerebrospinal fluid and serum in complex partial seizures. *Epilepsia* 1994;35:251-57.
8. Jhamandas K, Boegman RJ, Beninger RJ and Bialik M. Quinolinic acid-induced cortical cholinergic damage: modulation by tryptophan metabolites. *Brain Res.* 1990; 529: 185-191.
9. Leklem JE, Reynolds RD. Challenges and directions in the search for clinical applications of vitamin B-6. In: Leklem JE, Reynolds RD, ed. *Clinical and Physiological Applications of Vitamin B-6*, 1988: 437-54. *Current Topics in Nutrition and Disease*, V. 19; New York: Alan R. Liss, Inc.
10. Lumeng L, Li T-K, Lui A. The interorgan transport and metabolism of vitamin B-6. In: Reynolds RD, Leklem JE, ed. *Vitamin B-6: its Role in Health and Disease*. New York: Alan R. Liss, Inc. 1985: 35-54.
11. Lumeng L. Comments to Whyte MP, Mahuren JD, Vrabel LA, Coburn SP. Markedly increased circulating pyridoxal-5'-phosphate levels in hypophosphasia.

- Alkaline Phosphatase Acts in Vitamin B6 Metabolism. *J. Clin. Invest.* 1985; 76:752-6. *Hepatology* 1986; 6:330-1.
12. Luthman J, Vanerman E, Fredriksson G, Fornstedt-Wallin B. Regulation of quinolinic acid in the normal rat brain by kynurenine pathway precursors. In: Filippini GA et al., ed. *Recent advances in tryptophan research*. Plenum Press, New York, 1996:229-239.
  13. May CD. Vitamin B6 in human nutrition: a critique and an object lesson. *Pediatrics* 1954; 14:269-75.
  14. Metzler DE The chemical reactions in living cells. *Biochemistry, Academic Press*. New York, San Francisco, London, 1977.
  15. Perkins MN and Stone TW. An iontophoretic investigation of the actions of convulsant kynurenines and their interaction with the endogenous excitant quinolinic acid. *Brain Res.* 1982; 247: 184-187.
  16. Scriver CR, Beaudet AL, et al., ed. *The metabolic basis of inherited disease*. 6<sup>th</sup> Ed., New York: McGraw-Hill 1989; vol. 1,2.
  17. Schwarcz R, Speciale C, Okuno E, French ED and Kohler. Quinolinic acid: a pathogen in seizure disorders? In: Schwarcz R et al, ed. *Kynurenine and serotonin pathways. Progress in Tryptophan Research*. Plenum Press, 1991; 4: 697-707.
  18. Stone TW. Excitatory amino acids and dementia. In: Nicholson CD, ed. *Therapeutic Approaches to the Treatment of Dementia*. London, UK: Academic Press, 1993.
  19. Stone TW. Neuropharmacology of quinolinic and kynurenic acids. *Pharmacology Review* 1993; 45 (3) 310-379.
  20. Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, MacGregor GR. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nature Genetics* 1995; 11, september:45-51.
  21. Whyte MP, Mahuren JD, Fedde KN, Cole S, McCabe ERB, Coburn SP. Perinatal hypophosphatasia: tissue levels of vitamin B6 are unremarkable despite markedly increased circulating concentrations of pyridoxal-5'-phosphate. *J. Clin. Invest.* 1988; 81:1234-9.
  22. Yamamoto H. Studies on CSF tryptophan metabolism in infantile spasms. *Pediatr Neurol* 1991; 7: 411-4.

23. Yamamoto H, Horiguchi K, Egawa B, Murakami H. Studies on CSF kynureninase pathway abnormalities in patients with infantile spasms. *Ann Rep Jpn Epi Res Found* 1995; 7 172-7.
24. Young SN, Joseph MH, Gauthier S. Studies on kynurenine in human cerebrospinal fluid: lowered levels in epilepsy. *J Neural Transm* 1983; 58: 193-204.



Kynurenine pathway and related enzymes. Abbreviations:  
 IDO, indoleamine 2,3-dioxygenase; TDO: tryptophan 2,3-dioxygenase;  
 HAD: 3-hydroxyanthranilate 3,4-dioxygenase;  
 QPRT: quinolinate phosphoribosyltransferase

Figure 1

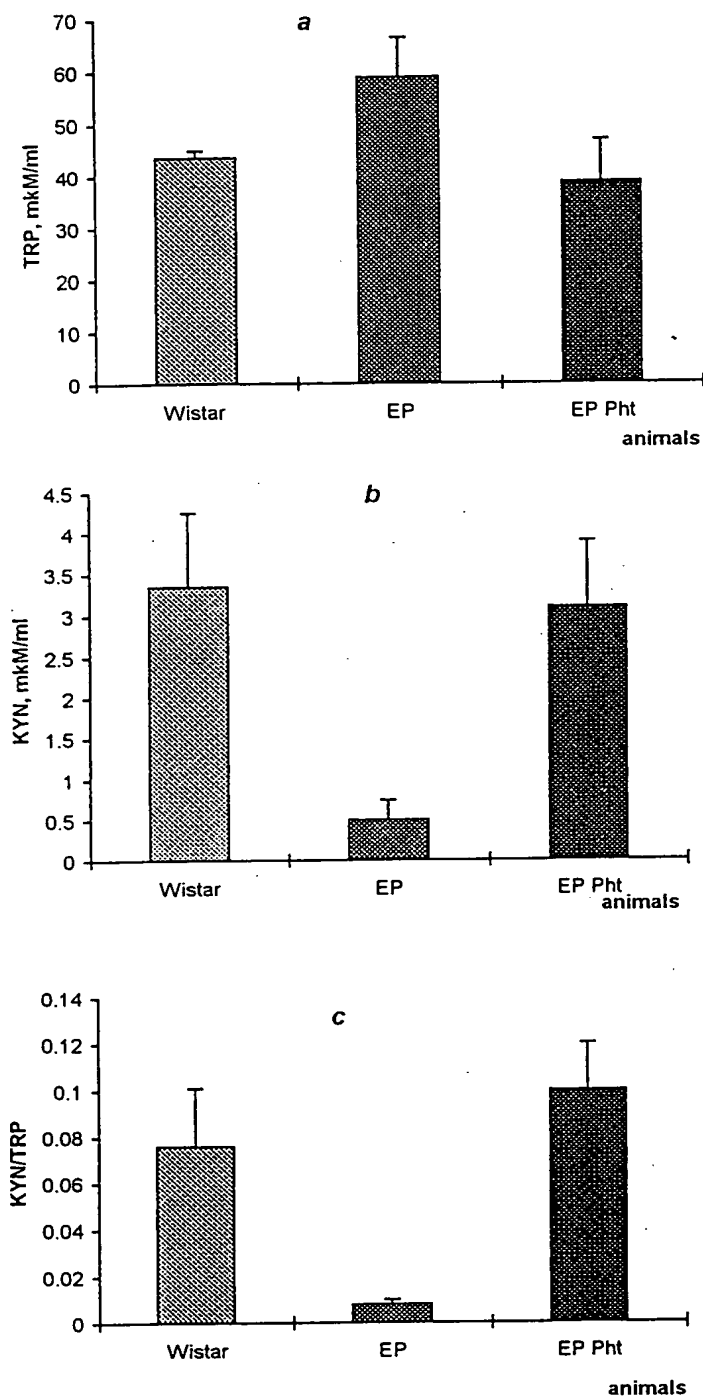


Fig 2. Plasma levels of tryptophan (TRP,*a*), kynurenine (KYN,*b*) and ratios of kynurenine to tryptophan (KYN/TRP,*c*) in epilepsy-prone (EP) rats in comparison with both epilepsy-resistant (ER) Wistar rats and EP rats chronically treated with phenytoin (EP Pht)

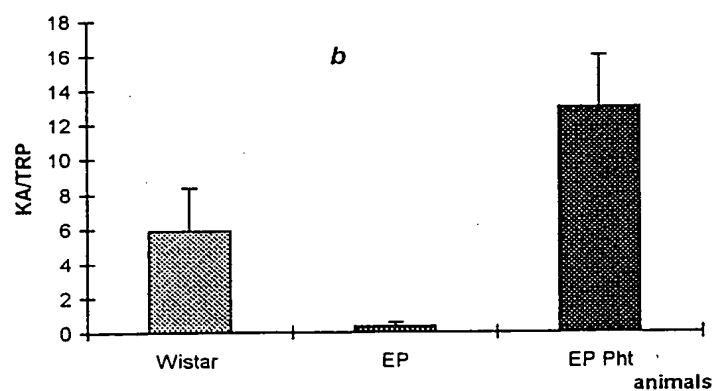
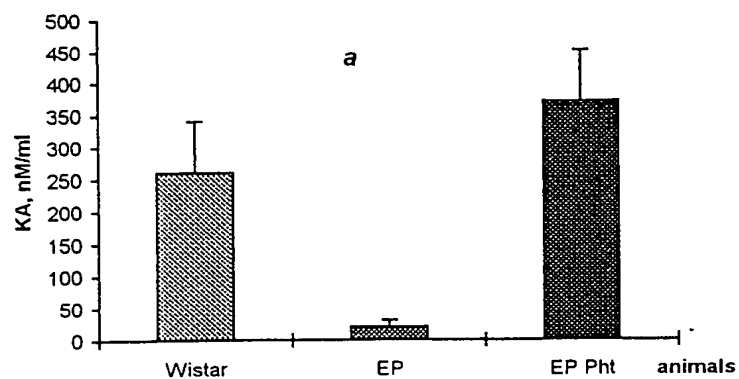


Fig 3. Plasma levels of kynurenic acid (KA,a) and ratios of kynurenic acid to tryptophan (KA/TRP,b) in epilepsy-prone (EP) rats, in comparison with epilepsy-resistant (ER) Wistar rats and EP rats chronically treated with phenytoin (EP Pht)

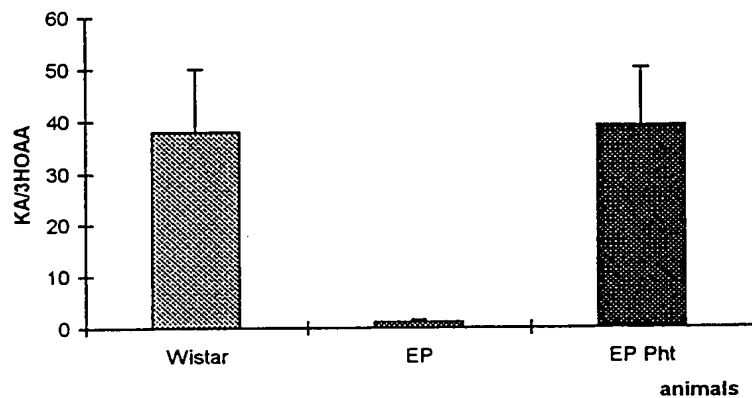


Fig 4. Ratio of kynurenic acid to 3-hydroxyanthranilic acid (KA/3HOAA) in epilepsy-prone (EP) rats in comparison with epilepsy-resistant (ER) Wistar rats and EP rats chronically treated with phenytoin (EP Pht)



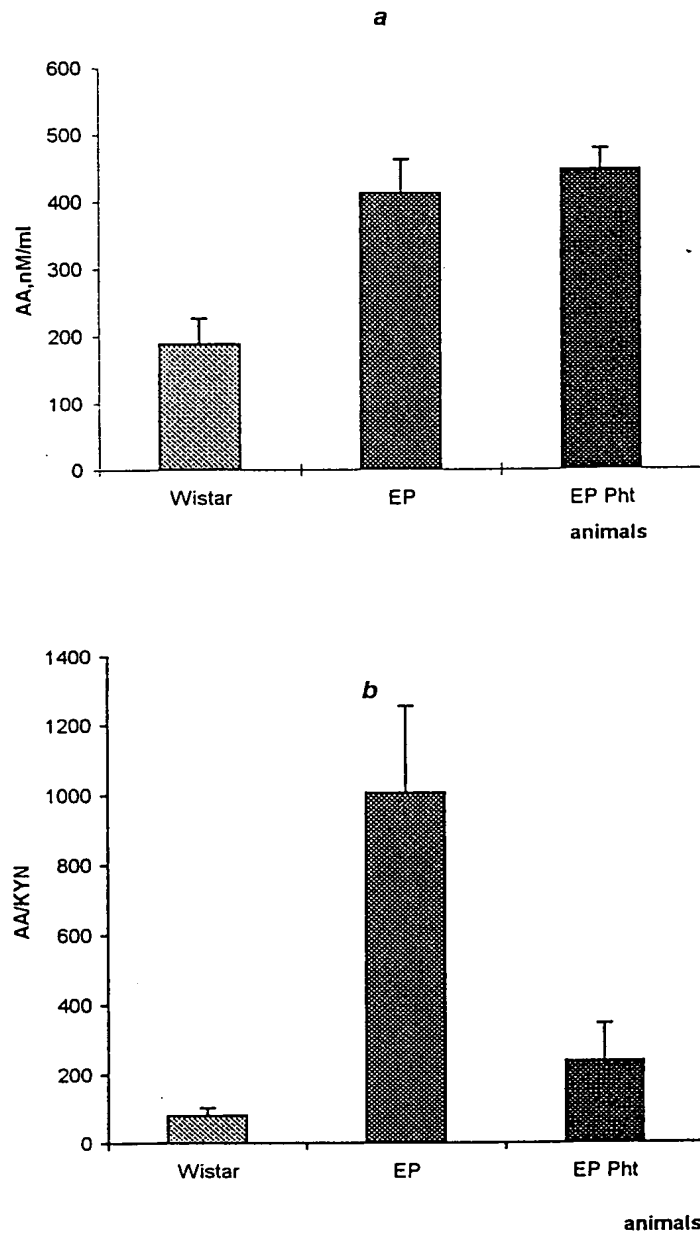


Fig 5. Plasma levels of anthranilic acid (AA,*a*) and ratio of anthranilic acid to kynurenine (AA/KYN,*b*) in epilepsy-prone (EP) rats, in comparison with epilepsy-resistant (ER) Wistar rats and EP rats chronically treated with phenytoin (EP Pht)

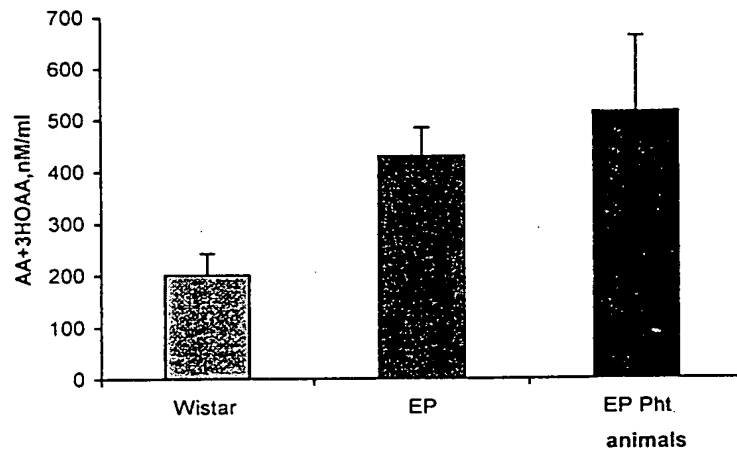


Fig.6 Total plasma levels of anthranilic and 3-hydroxyanthranilic acids (AA+3HOAA) in epilepsy-prone (EP) rats, in comparison with epilepsy-resistant (ER) Wistar rats and EP rats chronically treated with phenytoin (EP Pht)

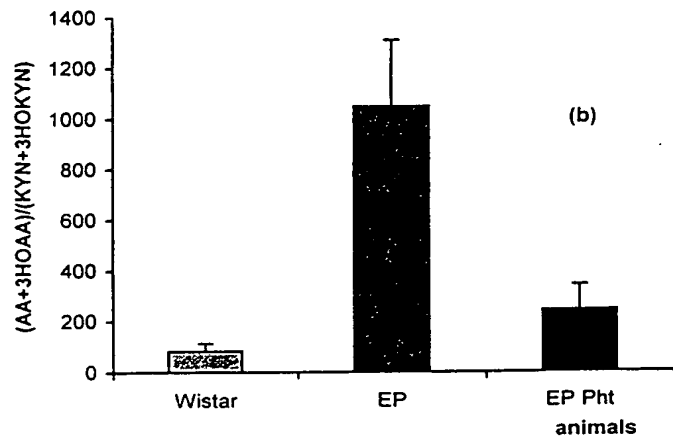
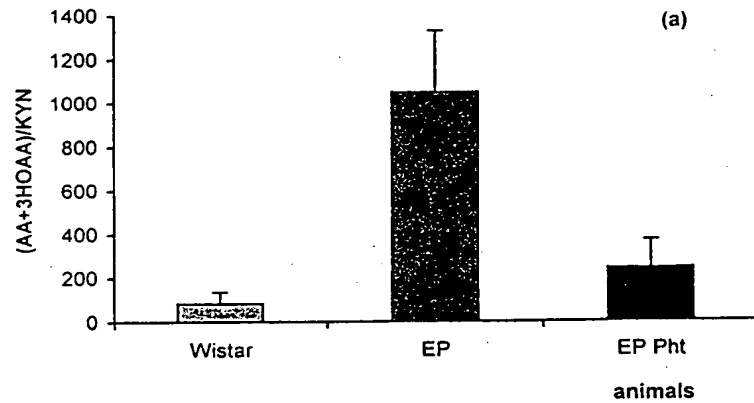


Fig.7 Ratio of total plasma anthranilates (AA+3HOAA) to kynurenine (KYN,a) and to the sum of kynurenines (KYN+3HOKYN,b) in epilepsy-prone (EP) rats, epilepsy-resistant (ER) Wistar rats and in EP rats chronically treated with phenytoin (EP Pht)

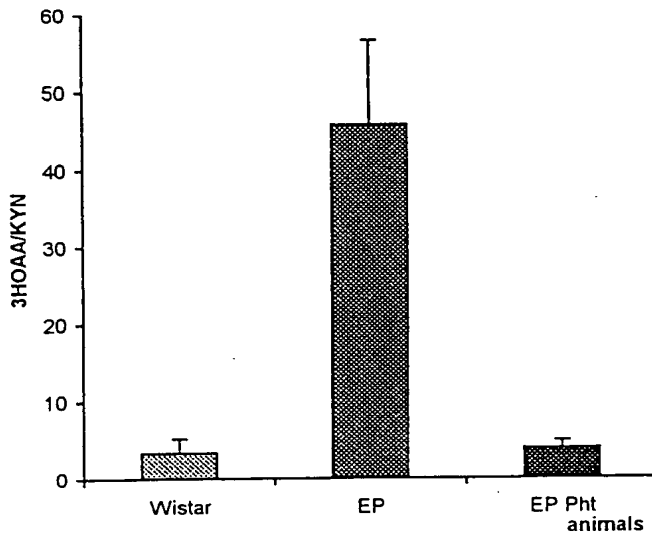


Fig. 8a Ratio of 3-hydroxyanthranilic acid (3HOAA) to kynurenine (KYN) in epilepsy-prone (EP) rats, in comparison with epilepsy-resistant (ER) Wistar rats and EP rats chronically treated with phenytoin (EP Pht)

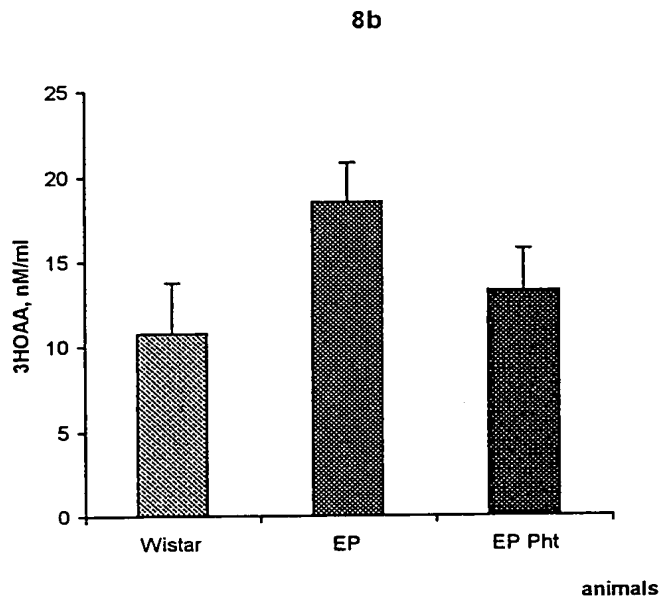
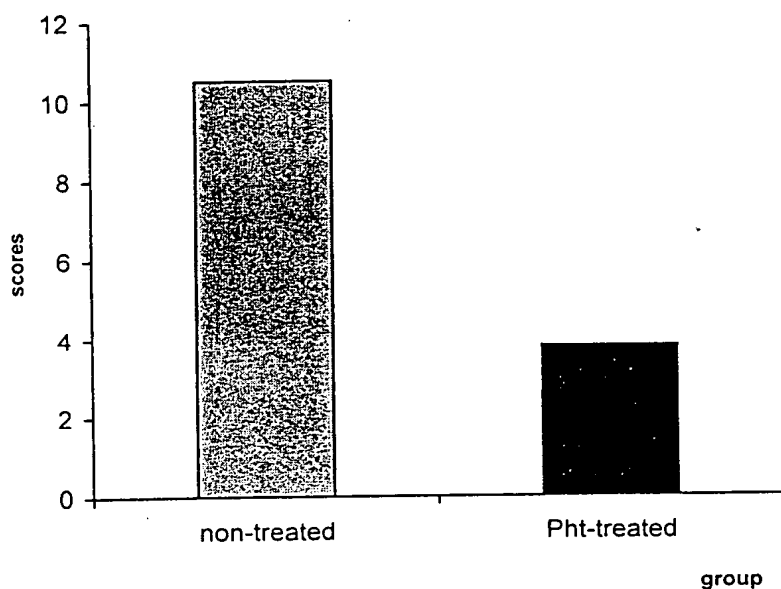


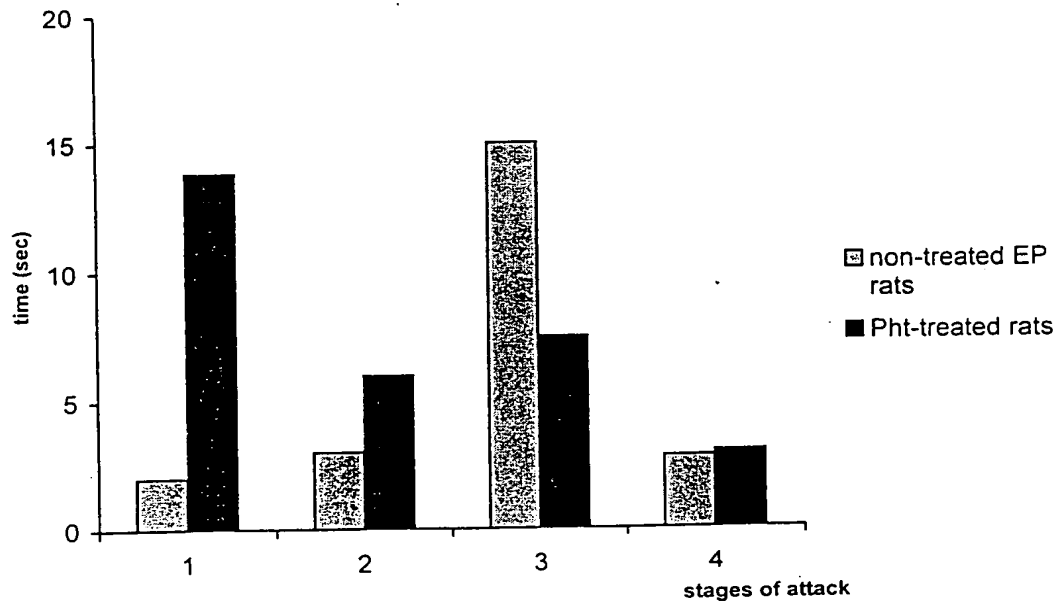
Fig. 8b Plasma concentrations of 3-hydroxyanthranilic acid (3HOAA) in EP rats in comparison with epilepsy-resistant Wistar (ER) rats and EP rats chronically treated with phenytoin (EP Pht)



**Scores:**

- 0 --absence of reactions
- 1 --short-term wild running
- 2 --two phase wild running
- 4 --tonic convulsions of flexors
- 6 --tonic convulsions of extensors
- 8 --postictal excitation or muscular atonia
- 10 --postictal excitation with convulsions
- 12 --postictal coma
- 16 --lethal exit

Fig. 9a Intensity of sound-induced convulsions (in scores) under chronic phenytoin treatment

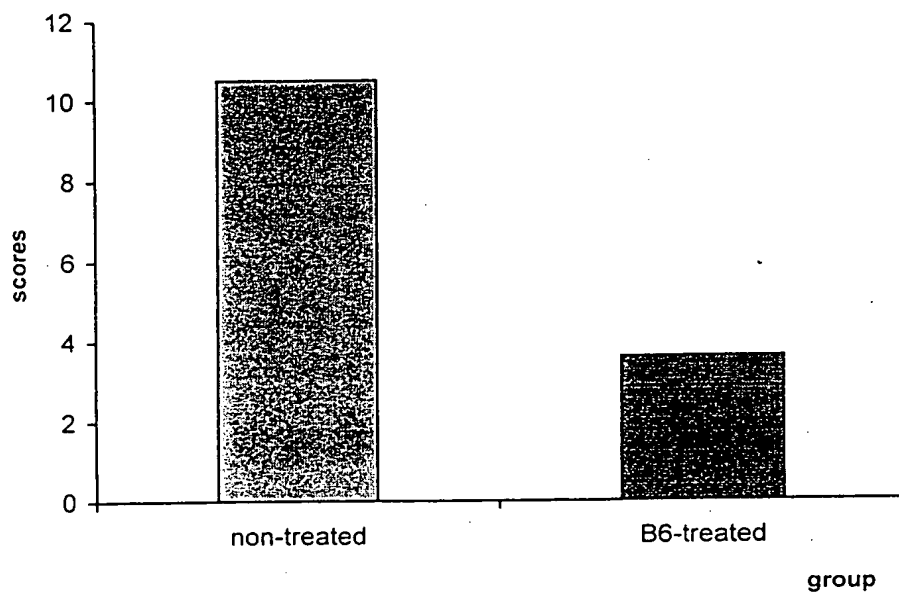


**Stages of attack:**

1. Latency
2. Wild running
3. Tonic convulsions of flexor or/and extensors
4. Clonic convulsions

Postictal condition	Non-treated	B6-treated
immobility	0/9	6/6
excitation	3/9	0/6
coma	6/9	0/6

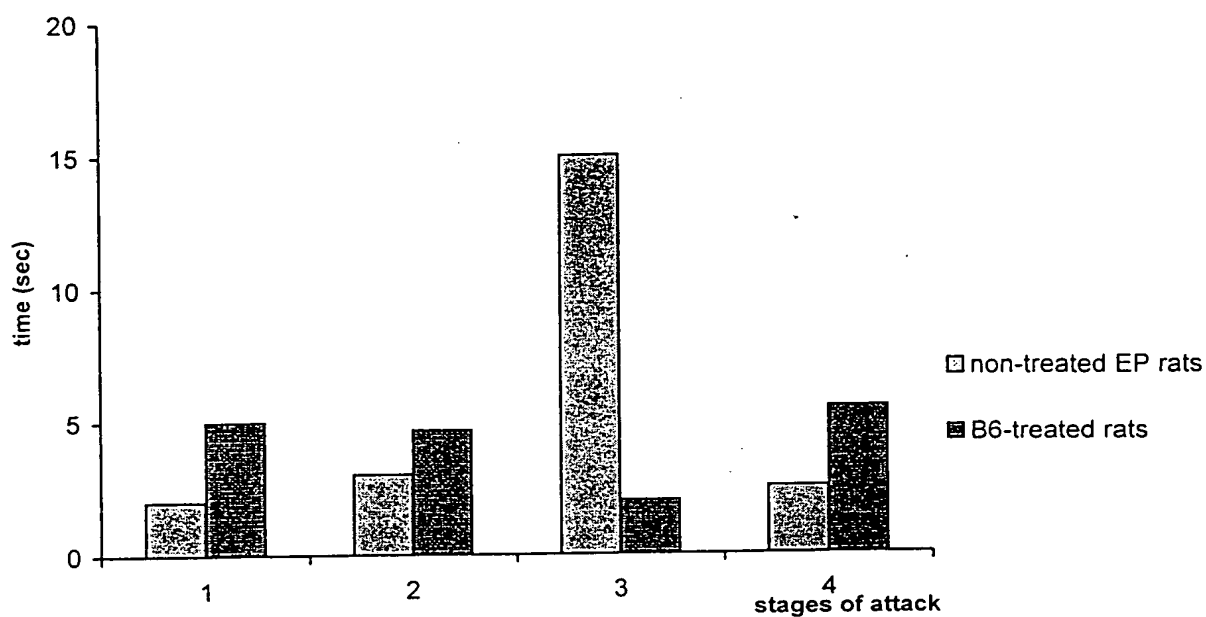
Fig. 9b Efficacy of chronic phenytoin (Pht): Sound-induced convulsions - duration of stages for rats having attacks



**Scores:**

- 0 --absence of reactions
- 1 --short-term wild running
- 2 --two phase wild running
- 4 --tonic convulsions of flexors
- 6 --tonic convulsions of extensors
- 8 --postictal excitation or muscular atonia
- 10 -postictal excitation with convulsions
- 12 -postictal coma
- 16 -lethal exit

Fig. 10a Efficacy of constant pyridoxine (B6) treatment: Intensity of sound-induced convulsions (in scores)



**Stages of attack:**

1. Latency
2. Wild running
3. Tonic convulsions of flexor or/and extensors
4. Clonic convulsions

Postictal condition	Non-treated	B6-treated
immobility	0/9	8/10
excitation	3/9	2/10
coma	6/9	0/10

Fig.10b Efficacy of constant pyridoxine treatment: Sound-induced convulsions - duration of stages

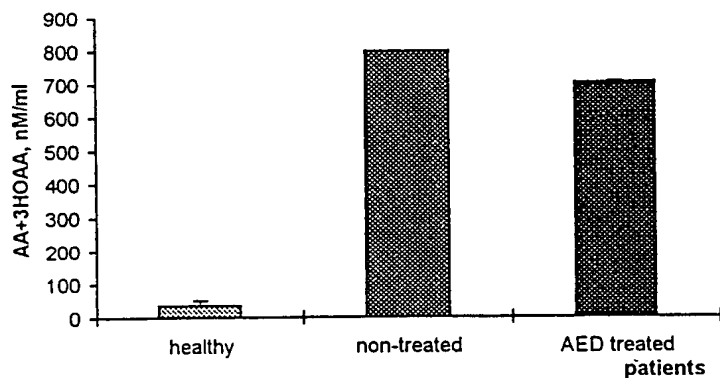


Fig. 11 Total sum of anthranilates (AA+3HOAA) in epileptic patients in comparison with healthy individuals

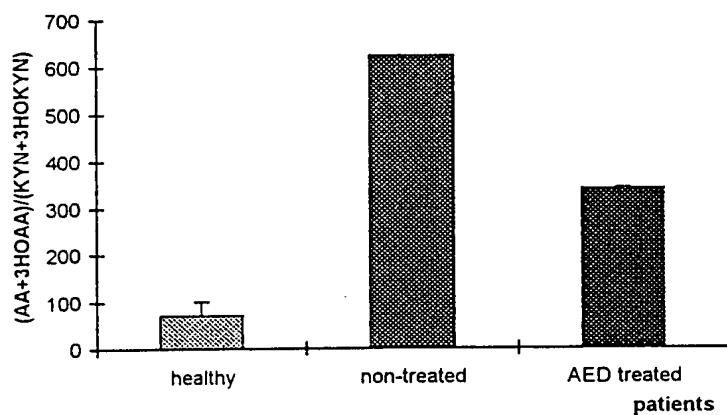


Fig. 12 Effectiveness of production of anthranilates (AA+3HOAA) from their precursors, KYN and 3HOKYN

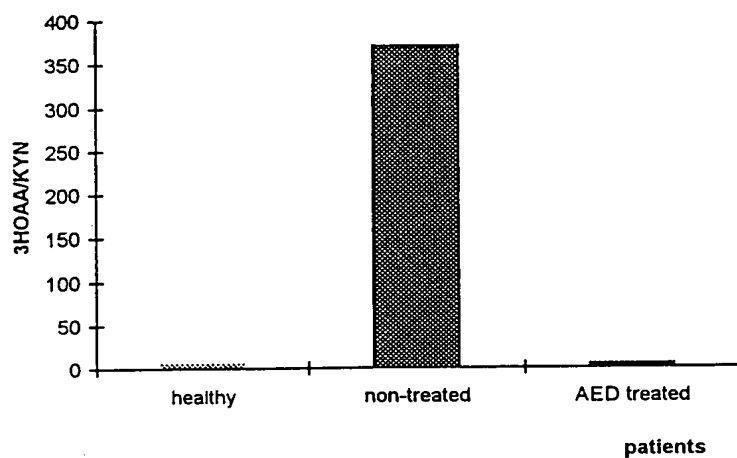


Fig. 13 Plasma ratios of 3HOAA to KYN in epileptic patients in comparison with healthy individuals



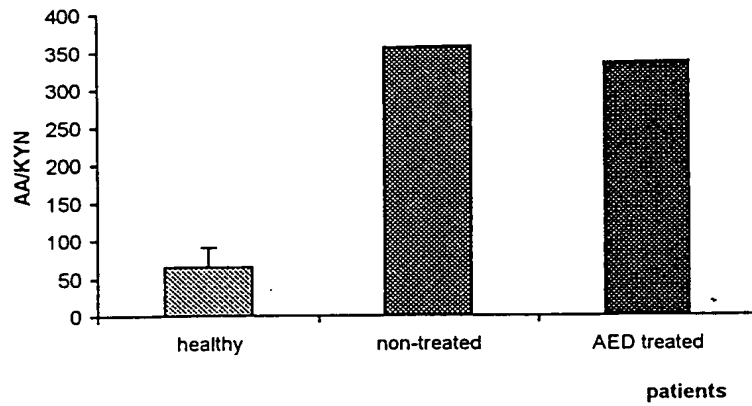


Fig. 14 Plasma ratios of AA to KYN in epileptic patients in comparison with healthy individuals

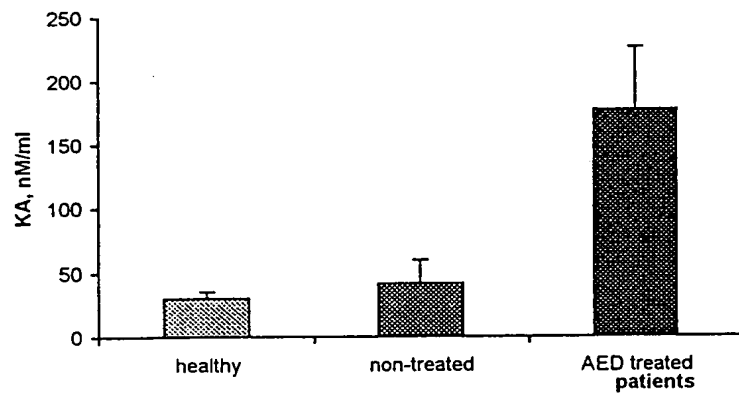


Fig. 15 Plasma levels of KA in epileptic patients in comparison with healthy individuals

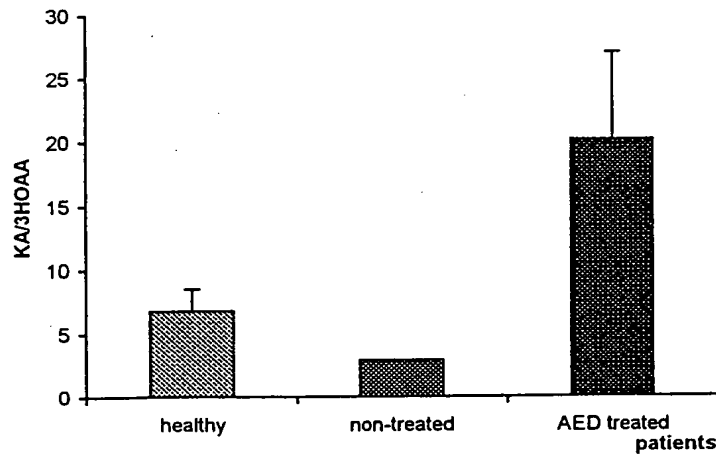


Fig. 16 Ratio of neuroprotective KA to neurotoxic 3HOAA in epileptic patients in comparison with healthy individuals

Brain tryptophan metabolites in epilepsy-prone rats in comparison with epilepsy-resistant Wistar rats (per 1.0 g of wet tissue)  
Effects of phenytoin (Pht) and pyridoxine (B6) treatment

*Cortex*

Metabolites	TRP	KYN	3HOKYN	3HOAA	AA	KA	3HOAA/TRP
	mkM	mkM	mkM	nM	nM	nM	
EP	49.0±3.26	(3/6)	< 0.01	374.7±35.9	202.6±43.6	282.1±124.1	7.6
EP/Pht	57.5±5.45	< 0.01	< 0.01	417.2±82.8	(3/6)	(1/6)	7.3
EP/B6	38.1±3.26	6.0 (4/6)	< 0.01	256.0±18.5	(1/6)	513.1 (4/6)	6.7
Wistar	42.5±7.62	1.63±1.2	< 0.01	271.2±40.3	< 1.0	(1/5)	6.4

*Midbrain*

Metabolites	TRP	KYN	3HOKYN	3HOAA	AA	KA	3HOAA/TRP
	mkM	mkM	mkM	nM	nM	nM	
EP	100.2±19.8	(2/5)	(1/5)	380.0±49.0	180.7±165.5	115.4	3.8
EP/Pht	144.5±44.2	< 0.01	< 0.01	465.0±185.3	< 1.0	(2/6)	3.2
EP/B6	75.8±11.7	(1/6)	(3/6)	418.4±44.3	< 1.0	< 1.0	5.5
Wistar	80.4±21.0	(1/6)	(2/6)	368.3±74.6	< 1.0	< 1.0	4.6

*Brain stem*

Metabolites	TRP	KYN	3HOKYN	3HOAA	AA	KA	3HOAA/TRP
	mkM	mkM	mkM	nM	nM	nM	
EP	84.1±15.8	0.39	0.11	221.6±25.5	69.7±11.6	(3/6)	2.6
EP/Pht	97.1±19.0	3.48	< 0.01	288.0±120.1	(3/6)	483	3.0
EP/B6	60.8±10.7	0.21	< 0.01	235.9±57.5	(1/6)	(3/6)	3.9
Wistar	104.0±11.4	0.56±0.23	(2/6)	288.0±52.0	< 1.0	(1/6)	2.8

*Cerebellum*

Metabolites	TRP	KYN	3HOKYN	3HOAA	AA	KA	3HOAA/TRP
	mkM	mkM	mkM	nM	nM	nM	
EP	40.8±8.3	0.38±0.3	0.07±0.055	36.4±18.0	(2/5)	81.1±52.2	0.9
EP/Pht	47.5±10.3	0.83	< 0.01	159.1±70.4	(3/6)	(1/6)	3.3
EP/B6	41.6±4.8	0.55±4.4	< 0.01	86.7±15.8	< 1.0	70.4	2.1
Wistar	41.6±1.2	0.16	(2/6)	98.9±28.1	< 1.0	< 1.0	2.4

Table 1

Table 3

The plasma tryptophan metabolites in non-treated epileptic patients

	TRP	KYN	3HOKYN	3HOAA	AA	KA	KYN/TRP	3HOAA/TRP	AA/TRP	KA/TRP	3HOAA/KYN	AA/KYN	KA/KYN	AA/3HOAA
Shira	32.6	0.69	0.0300	16.90	632.5	86.1	0.021	0.52	19.40	2.64	24.49	916.67	124.78	37.43
Leon(papa)	46.4	1.48	0.0180	9.35	620.6	54.2	0.032	0.20	13.38	1.17	6.32	419.32	36.62	66.37
Lev(son)	53.7	1.61	0.0001	23.90	281.0	40.0	0.030	0.45	5.23	0.74	14.84	174.53	24.84	11.76
Rom1	44.8	2.35	0.4400	41.10	1118.4		0.052	0.92	24.96		17.49	475.91		27.21
Rom2	42.0	1.05	0.0300	481.00	88.6	33.0	0.025	11.45	2.11	0.79	458.10	84.38	31.43	0.18
Gita	48.8	1.11	0.2300	2691.50	176.6	32.3	0.023	55.15	3.62	0.66	2424.77	159.10	29.10	0.07
N36	65.5	1.62	0.0001	12.70	272.4	36.9	0.025	0.19	4.16	0.56	7.84	168.15	22.78	21.45
T	18.9	0.77	0.0001		293	18.6	0.041		15.50	0.98		380.52	24.16	
N42	33.6	0.89	0.0001	6.70	391.4	32.0	0.026	0.20	11.65	0.95	7.53	439.78	35.96	58.42
Average	42.9	1.29	0.083	410.39	430.5	41.6	0.031	8.64	11.11	1.06	370.17	357.60	41.21	27.86
Disp	13.5	0.53	0.153											

Table 2

Plasma tryptophan metabolites in healthy humans

N	TRP	KYN	3HOKYN	3HOAA	AA	KA	KYN/TRP	3HOAA/TRP	AA/TRP	KA/TRP	3HOAA/KYN	AA/KYN	KA/KYN	AA/3HOAA
K1	30.2	0.40	0.00	6.05	37.3	47.3	0.0132	0.200	1.235	1.566	15.125	93.250	118.250	6.165
K2	32.8	0.53	0.00	0.00	24.6	29.0	0.0162	0.000	0.750	0.884	0.000	46.415	54.717	
K3	26.8	0.75	0.00	0.00	22.3	33.6	0.0280	0.000	0.832	1.254	0.000	29.733	44.800	
K4	32.0	1.18	0.00	5.00	62.1	40.5	0.0369	0.156	1.941	1.266	4.237	52.627	34.322	12.420
K5	28.0	1.01	0.00	8.70	47.0	32.9	0.0361	0.311	1.679	1.175	8.614	46.535	32.574	5.402
K6	25.5	0.28	0.00	0.00	27.6	19.2	0.0110	0.000	1.082	0.753	0.000	98.571	68.571	
K7	30.4	0.61	0.00	3.90	40.2	34.6	0.0201	0.128	1.322	1.138	6.393	65.902	56.721	10.308
K21	37.0	0.36	0.00	3.50	37.2	23.1	0.0097	0.095	1.005	0.624	9.722	103.333	64.167	10.629
K22	37.8	0.36	0.00	3.50	21.0	25.8	0.0095	0.093	0.556	0.683	9.722	58.333	71.667	6.000
K23	31.7	0.40	0.00	7.00	37.9	32.0	0.0126	0.221	1.196	1.009	17.500	94.750	80.000	5.414
K24	36.4	0.37	0.00	3.70	19.0	26.5	0.0102	0.102	0.522	0.728	10.000	51.351	71.622	5.135
K27	31.6	0.58	0.00	0.00	24.0	22.6	0.0184	0.000	0.759	0.715	0.000	41.379	38.966	
Average	31.7	0.57	0	3.45	33.35	30.6	0.0185	0.109	1.073	0.983	6.776	65.182	61.365	7.684
Disp.	2.36	0.17		1.79	7.7	4.8								

Continues... Table 3

KA/3HOAA	KA/AA	KA / 3HOAA+AA	KYN+3HOKYN/ 3HOAA	3HOKYN/ 3HOAA*1000	KYN/ AA *1000	AA+ 3HOAA	AA+3HOAA / KYN	KYN/ AA+3HOAA	(AA+3HOAA)/ (KYN+3HOKYN)
5.09	0.14	0.133	0.043	1.775	1.091	649.40	941.16	1.06	901.94
5.80	0.09	0.086	0.160	1.925	2.385	629.95	425.64	2.35	420.53
1.67	0.14	0.131	0.067	0.004	5.730	304.90	189.38	5.28	189.37
			0.068	10.706	2.101	1159.50	493.40	2.03	415.59
0.07	0.37	0.058	0.002	0.062	11.851	569.60	542.48	1.84	527.41
0.01	0.18	0.011	0.000	0.085	6.285	2868.10	2583.87	0.39	2140.37
2.91	0.14	0.129	0.128	0.008	5.947	285.10	175.99	5.68	175.98
	0.06	0.063			2.628	293.00	380.52	2.63	380.47
4.78	0.08	0.080	0.133	0.015	2.274	398.10	447.30	2.24	447.25
2.90	0.15	0.087	0.075	1.823	4.477	795.29	686.64	2.61	622.10
					3.375	824.59	745.78	1.77	607.35

Continue ... table 2

[illegible]

Table 4

### Kynurenine in the blood plasma of epileptic patients treated by AED

[illegible]

Continuation Table 4

KA/AA	KA / 3HOAA+AA	KYN+3HOKYN/ 3HOAA	3HOKYN/ 3HOAA*1000	KYN/ AA *1000	AA+ 3HOAA	AA+3HOAA / KYN	KYN*1000/ AA+3HOAA	KYN/AA+3HOKYN/3HOAA * 1000	(AA+3HOAA)/ (KYN+3HOKYN)
0.19	0.19	0.19	0.34	2.099	2027.85	479.40	2.09	2.10	479.40
0.98	0.95	0.08	0.000	2.178	349.50	472.30	2.12	2.18	472.30
0.30	0.29	0.19	0.000	5.026	388.16	204.29	4.89	5.03	204.29
0.41	0.40	0.16	0.000	2.815	744.37	361.34	2.77	2.81	361.34
0.16	0.16	0.24	0.000	1.750	1105.28	575.67	1.74	1.75	575.67
0.18	0.17	0.26	0.000	2.363	823.90	426.89	2.34	2.36	426.89
0.35	0.35	0.33	0.000	4.919	499.40	206.36	4.85	4.92	206.36
0.26	0.26	0.36	0.000	3.156	444.30	319.64	3.13	3.16	319.64
0.28	0.28	0.13	0.000	2.743	543.04	371.95	2.69	2.74	371.95
0.61	0.60	0.14	0.000	4.031	283.38	255.30	3.92	4.03	255.30
0.58	0.53	0.24	0.000	23.862	70.05	46.09	21.70	23.86	46.09
0.50	0.48	0.15	0.000	4.559	248.82	226.20	4.42	4.56	226.20
0.08	0.08	0.39	0.000	1.081	3090.18	927.98	1.08	1.08	927.98
0.29	0.28	0.13	0.000	3.956	301.81	260.18	3.84	3.96	260.18
1.19	1.11	0.08	0.000	5.342	163.93	199.91	5.00	5.34	199.91
0.48	0.46	0.22	0.000	6.787	233.80	151.82	6.59	6.79	151.82
0.43	0.41	0.21	0.000	4.792	707.36	342.83	4.57	4.79	342.83